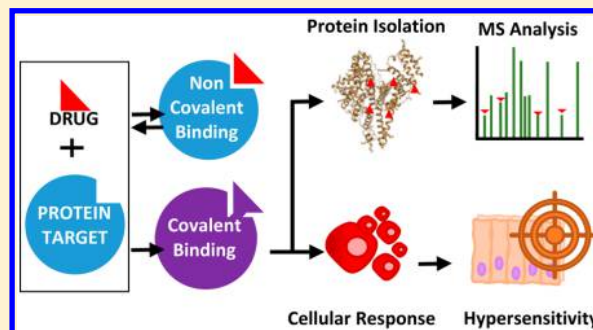


Mass Spectrometric and Functional Aspects of Drug–Protein Conjugation

Arun Tailor,[†] James C. Waddington,[†] Xiaoli Meng,^{*} and B. Kevin Park

MRC Center for Drug Safety Science, Department of Molecular and Clinical Pharmacology, University of Liverpool, Sherrington Building, Ashton Street, Liverpool L69 3GE, United Kingdom

ABSTRACT: The covalent binding of drugs (metabolites) to proteins to form drug–protein adducts can have an adverse effect on the body. These adducts are thought to be responsible for idiosyncratic drug reactions including severe drug hypersensitivity reactions. Major advances in proteomics technology have allowed for the identification and quantification of target proteins for certain drugs. Human serum albumin (HSA) and Hb have been identified as accessible targets and potential biomarkers for drug–protein adducts formation, for numerous drugs (metabolites) including β -lactam antibiotics, reactive drug metabolites such as quinone imines (acetaminophen) and acyl glucuronides (diclofenac), and covalent inhibitors (neratinib). For example, MS/MS analysis of plasma samples from patients taking flucloxacillin revealed that flucloxacillin and its 5-hydroxymethyl metabolite formed covalent adducts with lysine residues on albumin via opening of the β -lactam ring. Other proteins such as P450 and keratin are also potential targets for covalent binding. However, for most drugs, the properties of these target proteins including their location, their quantity, the timing of conjugate generation, and their biological function are not well understood. In this review, currently available proteomic technologies including MS/MS analysis to identify antigens, precise location of modifications, and the immunological consequence of hapten–protein complex are illustrated. Moving forward, identification of the nature of the antigenic determinants that trigger immune responses to drug–protein adducts will increase our ability to predict idiosyncratic toxicity for a given compound.



CONTENTS

1. Introduction	B	3.2. Direct Toxicity	N
2. Mass Spectrometric Characterization and Quantification of Drug–Protein Adducts	B	3.2.1. Liver Toxicity	N
2.1. Detection of Drug–Protein Adducts	B	3.2.2. Mechanism Based Inhibition	N
2.1.1. Sample Isolation	C	3.3. Immunological Activation	O
2.1.2. Radiometric Detection	C	3.3.1. Immediate Hypersensitivity Reactions	O
2.1.3. Drug–Protein Adducts Detection Using Antibodies	D	3.3.2. Mechanism of Delayed Type Hypersensitivity	O
2.2. Mass Spectrometry Based Proteomics for Qualitative and Quantitative Analysis of Drug–Protein Adducts	E	3.3.3. Role of the Innate Immune System in Drug Hypersensitivity	P
2.2.1. Top-down Mass Spectrometric Analysis	E	3.3.4. Role of Adaptive Immunity in Drug Hypersensitivity	P
2.2.2. Bottom-up Mass Spectrometric Analysis	F	3.3.5. Advances in Understanding Immune-Mediated Adverse Reactions	Q
2.2.3. Quantification	G	4. Discussion	Q
2.3. Protein Adducts as Biomarkers for Exposure to Reactive Drug Metabolites	G	Author Information	R
2.3.1. β -Lactam Antibiotics	H	Corresponding Author	R
2.3.2. Arene Oxides and Epoxides	I	Author Contributions	R
2.3.3. Michael Acceptors	K	Funding	R
2.3.4. Aromatic Amines	L	Notes	R
2.3.5. Acyl Glucuronides	M	Biographies	R
3. Functional Aspects	N		
3.1. Understanding the Function of Drug–Protein Adducts	N		

Special Issue: Mass Spectrometry and Emerging Technologies for Biomarker Discovery in the Assessment of Human Health and Disease

Received: May 1, 2016

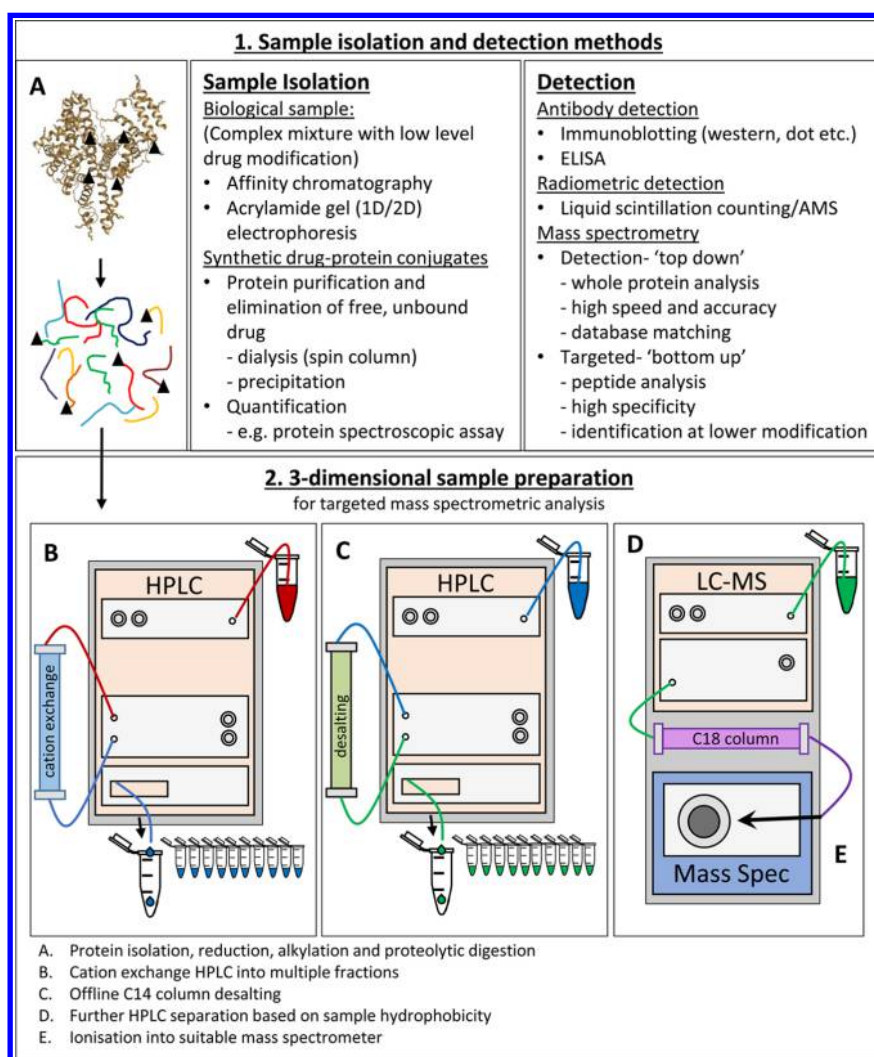


Figure 1. An overview of (1) the methods utilized in the isolation and detection of drug–protein adducts and (2) 3-dimensional sample preparation (A–E) for the enhancement of acquisition sensitivity.

Abbreviations
References

R
S

drug–protein adduct formation.⁷ This review will describe advances in MS technology, define the chemistry of adduct formation, and highlight the challenges in identifying the functional role of these adducts in adverse drug reactions (ADRs).

1. INTRODUCTION

A drug–protein adduct is a distinct molecular species resulting from the reaction of a protein and a chemically reactive drug (metabolite). The covalent modification of proteins can play an essential role in the pharmacological activity of a wide range of drugs.^{1–3} However, covalent modification of proteins can also lead to drug toxicity through several mechanisms including the induction of an unwanted immune response and drug hypersensitivity. Determining the target proteins of these drug molecules or reactive metabolites along with the function of these adducts has been difficult to discern.

The discovery that small chemical compounds could initiate toxicity by becoming covalently bound to proteins arose indirectly from studies of the metabolism of carcinogenic aminoazo dyes in rats.⁴ Subsequent studies demonstrated that drug–protein adducts could play a role in drug hypersensitivity reactions with the extensive research conducted on penicillins and dinitrophenyl compounds.^{5,6} Recent advances in mass spectrometry (MS) have significantly aided our understanding of

2. MASS SPECTROMETRIC CHARACTERIZATION AND QUANTIFICATION OF DRUG–PROTEIN ADDUCTS

Different drugs have the ability to bind proteins in different ways. Identifying the precise chemical nature of drug–protein adducts is an important first step in understanding mechanisms of drug hypersensitivity. Novel mass spectrometric methods have been developed for the identification and quantification of drug–protein adducts, even when formed at very low concentration. This section gives an overview of these techniques followed by a more detailed chemical outline of the types of adducts which have been found.

2.1. Detection of Drug–Protein Adducts. Multiple methods are used to detect drug–protein adducts (Figure 1). The most suitable method is dependent on type of sample, the required specificity and sensitivity, and the depth of analysis needed. While MS techniques now dominate drug–protein

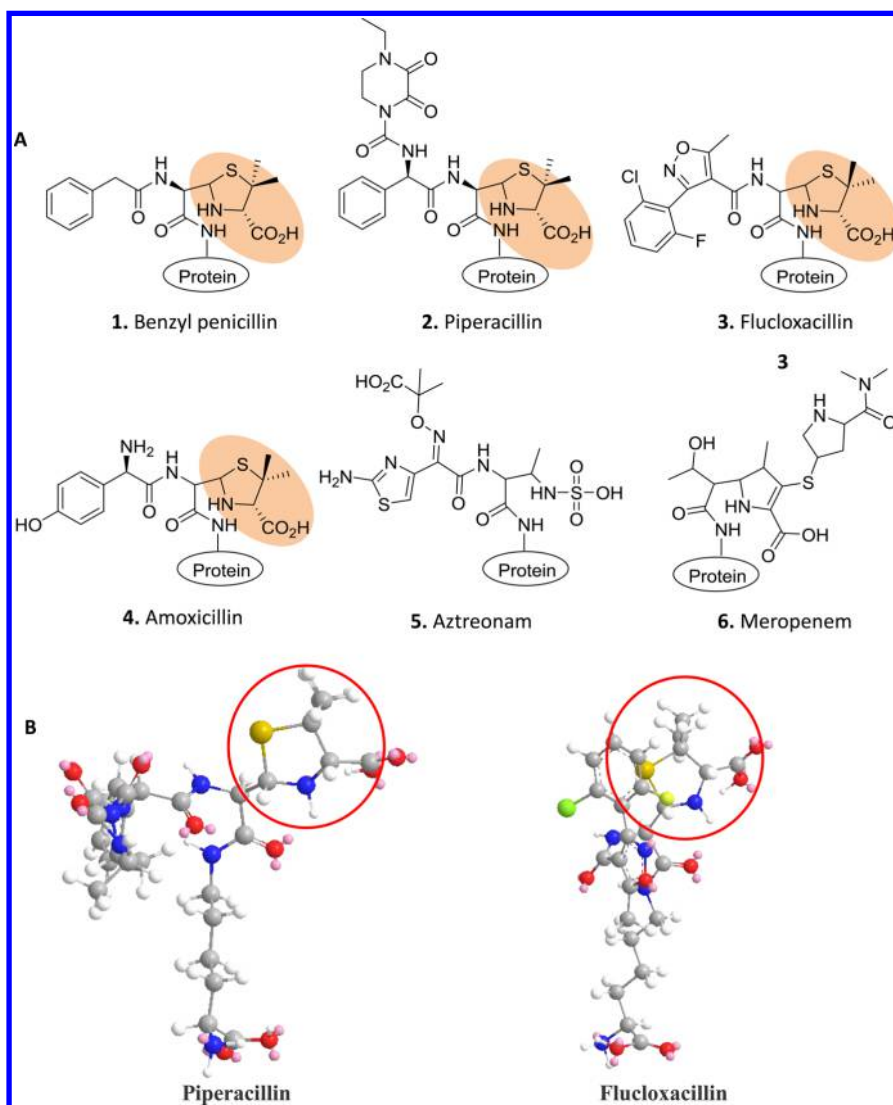


Figure 2. Haptenic structures of the β -lactam–protein complex. Nucleophilic addition to β -lactam ring by lysine residues on proteins results in ring opening and formation of a stable amide adduct (A); 3D models show that the thiazolidine ring of piperacillin–lysine is exposed for antibody recognition, whereas it is masked by side chains in flucloxacillin–lysine conjugates (B).

adduct analysis, traditional methods of protein purification are still essential.

2.1.1. Sample Isolation. Biological samples such as blood serum contain an extremely complex mixture of proteins, estimated to consist of more than 10,000 proteins spanning 9 orders of magnitude in concentration.⁸ Therefore, the identification of target proteins conjugated by drugs is a challenge. Highly abundant proteins, including HSA, account for 80% of the total protein concentration in serum.⁹ HSA is often targeted by many drugs (metabolites) both due to its high abundance and its ability to nonspecifically and reversibly bind to many lipophilic organic compounds.^{10,11} Additionally, HSA is particularly susceptible to modification by β -lactam antibiotics, which bind covalently to available lysine residues.^{12–16} Isolating individual protein adducts for mass spectrometric analysis can be achieved using both HPLC methods (protein depletion and sample fractionation) and SDS–PAGE. During protein depletion, a HPLC column containing an affinity antibody/ligand modified resin can be used to isolate target proteins from biological samples.^{17,18} Alternatively, HPLC fractionation by

protein hydrophobicity can be utilized in the analysis of complex samples (Figure 1).

The *in vitro* incubation of drugs (metabolites) with model proteins such as HSA and glutathione *S*-transferase pi (GSTP) has given insight into the chemical mechanisms of drug–protein adducts formation. For example, the chemical nature, the level of modification, and the stability of adducts can be investigated by altering incubation conditions such as buffer pH, drug concentrations, and incubation times. By altering these conditions, it is possible to generate sufficient quantities of drug–protein adducts to determine the structures of potential adducts and possible chemical pathways for the adducts which might be formed *in vivo*. Such *in vitro* studies are required for the development of methods with sufficient sensitivity for analysis of drug–protein adducts formed in low abundance from biological samples.^{13,18,19} Following the incubation of drug with protein, it is essential that unbound drug is removed from the sample. The protein is then analyzed using SDS–PAGE and Western blotting using drug specific antibodies.

2.1.2. Radiometric Detection. The use of radiolabeled chemicals is essential for understanding drug disposition during

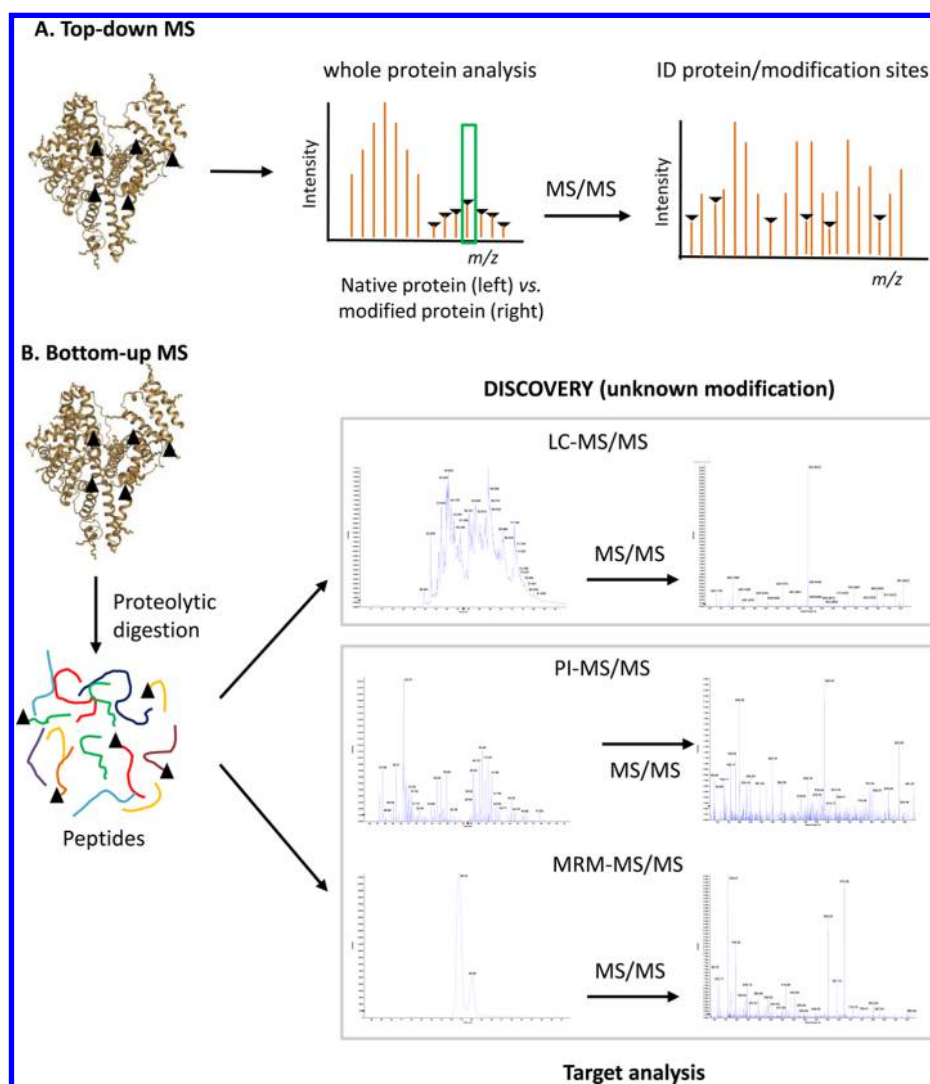


Figure 3. Top-down (A) vs bottom-up (B) MS method for the characterization of drug–protein adducts. In top down MS, the whole protein is directly analyzed by MS without digestion, and further fragmentation will enable one to locate the modification sites (A);³¹ in bottom-up MS, proteins are digested by an enzyme into peptides that can be analyzed by various MS techniques: MS/MS analysis allows for identification of unknown adducts; more targeted analyses (precursor ion-MS/MS and multiple reaction monitoring-MS/MS) enable one to detect known adducts with great sensitivity (B).

drug development.^{20,21} Most pharmaceutical companies synthesize isotopically labeled compounds for covalent binding studies at the lead optimization stage of drug discovery. After both *in vitro* or *in vivo* studies, radioactive drugs covalently bound to proteins can be detected using accelerated mass spectrometry (AMS) or liquid scintillation counting (LSC).²² While LSC is perhaps the least complex and cheapest method, limits of detection and light frequency disruptions may hamper identification of radioactive material at low concentrations. Although AMS does enable more precise analysis from smaller sample volumes, instrumental platforms are very expensive.²³ Therefore, radiometric analysis in combination with SDS–PAGE is the most widely used method for the detection of drug–protein adducts in complex systems.^{24–26} Although there are clear advantages to using radiometric detection, the cost and storage/disposal of radioactive material make its use in most research facilities limited.

2.1.3. Drug–Protein Adducts Detection Using Antibodies. Antibodies specific to particular molecular targets can be used to detect drug–protein adducts through a variety of techniques, including immunoblots and ELISAs. While these are very coarse

methods of hapten detection, they represent a simple way to quickly detect molecules of interest. In addition, methods used to separate complex mixtures, through 1-D and 2-D SDS–PAGE and HPLC, can be used in conjunction with antibodies to identify drug–protein adducts. Immunoblots are highly qualitative and are best used to visually display a set of data. On the contrary, absorbance readings from ELISA samples provide quantitative measurement which can be statistically analyzed allowing confidence values to be calculated.

Drug specific antibodies are used for the detection of drug modified proteins. While this practice is widely accepted in the field, there are limitations. As immunoblotting and ELISAs rely on antibodies that recognize a specific epitope, cross-reactivity can be problematic. Figure 2A demonstrates how chemical moieties are conserved within drug families, such as the thiazolidine ring in β -lactam antibiotics.^{19,27} Although the thiazolidine ring is conserved across the family of β -lactam antibiotics, penicillin antibodies are not cross-reactive with all members. Commercially available antibodies raised to benzylpenicillin that recognizes the thiazolidine ring do not cross-react with other β -lactams such as flucloxacillin, oxacillin, cloxacillin,

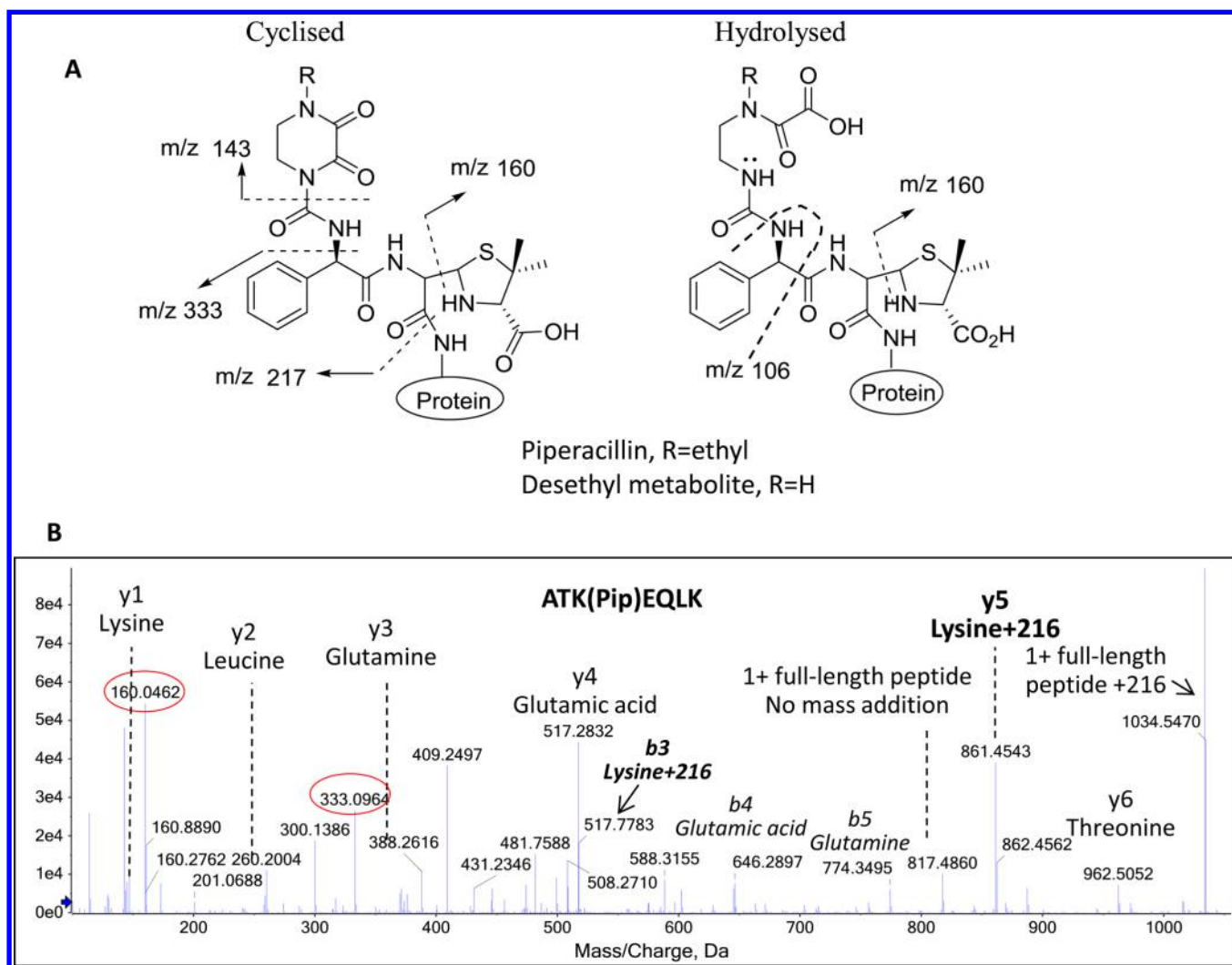


Figure 4. MRM-MS/MS methods for the detection of drug-modified proteins. In patients, piperacillin forms four types of haptenic structures with lysine residues on albumin; each generates a characteristic fragmentation ion of m/z 160 amu that was used in MRM-MS/MS for the detection of piperacillin-modified albumin (A); MS/MS spectrum of the piperacillin-modified tryptic peptide $^{539}\text{ATK}^*\text{EQLK}^{545}$ with piperacillin attached to Lys541(B).⁶¹

and dicloxacillin. Although these all possess a thiazolidine ring, the thiazolidine rings in these molecules have been masked by the isoxazole group present in these β -lactams due to the steric hindrance, thus preventing antibody recognition (unpublished data, Waddington et al. 2016) (Figure 2B).

2.2. Mass Spectrometry Based Proteomics for Qualitative and Quantitative Analysis of Drug–Protein Adducts. While all of the aforementioned techniques have the ability to detect drug–protein adducts, more sophisticated tools are required to characterize the precise chemical nature and for the identification of novel drug–protein adducts. MS is an exceptionally powerful tool used to elucidate structures of both known and unknown adducts.²⁸ Through peptide sequencing with MS techniques, it is possible to determine the precise amino acid modification in the protein structure. In the analysis of peptides using MS following ionization, mass-to-charge ratios (m/z) of peptides are calculated. Tandem MS utilizes a second step to enable further fragmentation and analysis of the resulting product ions and is the tool of choice when sequencing peptides.²⁹ MS platforms have also been developed to perform differently based on sample type and expected results. A “trade-off” between sensitivity and mass accuracy can be observed between different MS platforms,²⁸ where depending on the

sample mixture one may be favored more than the other. This section will outline the main platforms of MS analysis used for the detection and quantification of drug–protein adducts and their limitations in particular sample types.

2.2.1. Top-down Mass Spectrometric Analysis. As protein targets for many drugs (metabolites) in complex biological samples (e.g., blood plasma) remain unknown, less targeted MS approaches are required for adducts discovery. Top-down MS works by introducing whole proteins into the mass spectrometer where the molecular weight of an intact protein is first identified (the “top” part, Figure 3A).³⁰ Further fragmentation by tandem MS (the “down” part) allows identification of proteins with full sequence coverage. Since top-down MS analyses intact proteins without proteolytic digestion, this strategy is extremely useful for mapping of labile drug–protein adducts that may be lost during digestion or cleaved off in MS analysis.³¹

Top-down MS is generally performed in high resolution instruments because of the need to resolve the high molecular weight of intact proteins.³¹ Historically, Fourier transform ion cyclotron resonance mass spectrometers have been mostly used for top-down MS analysis. More recently, new instruments with a high resolving power that facilitate tandem MS experiments have been developed.³² For example, QTOF (quadrupole time-of-

flight) MS/MS platforms can simultaneously offer both speed and accuracy, allowing for large amounts of data to be obtained from complex samples.³³ In proteomics, thousands of highly accurate spectra can be produced relatively quickly. Consequently, data can be matched to protein databases in order to identify proteins present in the sample. Certain sample mixtures are too fragile and will therefore fragment during initial ionization,^{34,35} leading to reduced spectra acquisition on typical quadrupole mass spectrometers. Matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) MS is used with these samples.^{34,35} This “soft” ionization technique causes little or no sample fragmentation, leading to increased resolution of analyte ions.^{34,35}

Top-down MS is used for the detection of drug modified adducts from complex sample mixtures (Figure 3A). Initially, the protein of interest will be isolated from the sample. This can be achieved using HPLC, either through protein depletion or fractionation. While protein depletion using affinity capture is generally more preferred, this is limited by the availability of antibodies/ligands to proteins of interest. On analysis, drug–protein adducts will gain a mass addition, depending on the level of modification, resulting in a mass-shift that would be identifiable on the mass spectra produced (Figure 3A). The incorporation of novel MS fragmentation techniques such as electron capture dissociation, has also allowed for the identification of modification sites.^{32,36} Although top-down MS has proven to be a powerful technique for the identification of the sites of protein modification and determination of the order of multiple modification, several technical challenges such as protein solubility and sensitivity are yet to be resolved.³¹ An alternative bottom-up MS approach, involving proteolytic digestion of proteins into small peptides prior to MS analysis, has been widely used for identification and quantification of drug–protein adducts.

2.2.2. Bottom-up Mass Spectrometric Analysis. The introduction of peptides into a mass spectrometer is known as a “bottom-up” approach. In this method, samples undergo reduction and alkylation, using dithiothreitol and iodoacetamide, respectively, to unfold the tertiary protein structure and prevent refolding. Samples are subsequently digested using proteolytic enzymes (Figure 3B).^{37,38} Similar to top-down analysis, proteins must first be extracted from the sample mixture using the same methods. Alternatively, in bottom-up analysis proteins of interest can be isolated by SDS–PAGE followed by in-gel digestion of the bands of interest. It is advantageous for samples to undergo desalting, concentration, and purification with a suitable C18 filter column in order to enhance the sensitivity of detection.^{39,40} Samples are then loaded into the mass spectrometer as a solution of digested peptides.

In the discovery stage, QTOF MS/MS is still a powerful platform used for the detection and analysis of unknown drug–protein adducts. Product ion scanning is a nontargeted approach where the initial ion (precursor ion) is detected in the first ionization stage and then further fragmented to detect the full peptide sequence. Although this is a nonspecific scanning technique, modern QTOF platforms can achieve up to 25 MS/MS spectra per cycle, providing masses of sample data for analysis. Similarly, precursor ion scanning can be used to identify suspected modifications in an unknown sample (Figure 3B). In precursor ion scan mode, particular precursor ions that produce a preselected product ion will be detected, generating a record of analytes that give clues to potential drug modifications. For example, a characteristic peak at m/z 160, corresponding to the

cleavage of the thiazolidine ring, can be used for identifying adducts formed by β -lactam antibiotics (Figure 4A).^{16,18} This type of experiment is extremely useful for monitoring unknown protein targets for known adducts within a complex mixture. While this is a partially targeted approach to drug–protein adducts detection, it can give rise to false positives. Ions that are not derived as a result of drug cleavage may correspond to the same mass; therefore, it is important to manually characterize the amino acid sequence on any spectra where drug modification is suspected. Putative drug modified peptide sequences can be matched against peptide databases with the hope of identifying protein origins. Although in theory this is a promising method to identify novel drug–protein adducts, in practice it is laborious and takes a trained eye to calculate peptide sequences.

Multiple reaction monitoring (MRM) is a more targeted MS tool enabling the user to scan for specific parent and fragment ions. MRM transitions specific for drug modified peptides are generally designed for this purpose. The parent ions can be calculated for all possible peptides derived from theoretical digests with a mass addition of proposed haptens. In some cases, peptides with missed cleavage sites are commonly used when drugs (metabolites) are covalently bound to lysine or arginine. The parent ion masses are then paired with the proposed fragment masses derived from drug molecules. MRM-MS/MS actively searches for predefined m/z ratios during data acquisition, and further fragmentation is only performed on the ions that match the search criteria.⁴¹ Instead of obtaining full MS/MS analysis, only a small number of specific fragment ions are analyzed, making analysis more specific and sensitive. By monitoring of the specific ions of interest, MRM-MS/MS scanning has the capability to detect extremely low levels of drug-modified proteins present in the complex biological matrices (Figure 3B).⁴²

MRM is particularly useful in the identification of proteins modified by β -lactam antibiotics. Protein digestion using known enzymes, such as trypsin, enables theoretical digests and expected m/z ratios of peptides to be calculated. β -Lactam antibiotics covalently bind to exposed NH_2 groups on the side chains of lysine residues.⁴³ When a drug is covalently bound, proteolytic digestion of trypsin cleaving the C-terminal side of lysine and arginine is interrupted.⁴⁴ This is due to the chemical morphology at a β -lactam antibiotic bound lysine residue blocking chemical interactions, thus incorporating missed cleavage sites.^{18,45} Missed lysine cleavages are therefore included when performing theoretical digests. This enables the specific selection of peptides with the potential to be modified at a nonterminal lysine residue. To specifically search for drug modified peptides, the m/z ratios are calculated with a mass addition corresponding to the molecular weight (MW) of haptens. When analyzing the spectra, characteristic features of the drug molecule can be identified, for example, the cleavage of thiazolidine ring of penicillin leads to the fragment ion at m/z 160.^{16,18}

Sample preparation is important in being able to successfully identify drug–protein adducts, especially when they are present in low abundance. Therefore, a three-dimensional approach to sample preparation is performed. Proteins of interest can be first isolated from complex biological samples using affinity capture and subjected to proteolytic digestion. After digestion, HPLC platforms are used for ion exchange, separating samples into multiple fractions depending on their ionic interactions with the column used. Each fraction will then be purified to remove the salt using C18 reverse phase HPLC, before MS analysis. Sample

Table 1. Drug Modified Proteins Detected *in Vitro* and *in Vivo*

drugs	reactive motif	targeted proteins		modified AA	adducts formed	ref
		<i>in vitro</i>	<i>in vivo</i>			
penicillin	beta-lactam	albumin	albumin	Lys	amide	13,16,18
diclofenac	acyl glucuronide quinone imine	GSTP, albumin	albumin	Lys, Cys	Schiff base thioether	105,175
abacavir	enone	GSTP, albumin	albumin	Lys, Cys, His	Michael adducts	83,84,176
DNCB ^a	aryl halide	K14 albumin	NA	Lys, Cys, Tyr	aromatic substitution	177,178
acetaminophen	quinone imine	GSTP, albumin, CP2C6_RAT, MADD_RAT, MGST1_RAT, NU5M_RAT	albumin	Cys	thioether	74,77,179
nevirapine	arene oxide quinone methide, sulfate	GSTP, albumin, Hb	Hb, albumin	Cys, His, Val	thioether Michael adducts	69,70,72,82
carbamazepine	arene oxide epoxide	GSTP, albumin	albumin	Cys, His	thioether	65
isoniazid	hydrazine	albumin	albumin	Lys	amide	180
MRL-A ^a	α,β -unsaturated imine	liver and kidney proteins	acyl-CoA synthase	Lys, Ser, Cys	Michael adducts	181
ibrutinib	α,β -unsaturated amide	albumin	NA	Cys, Lys	Michael adducts	182
SMX ^a	Nitroso	GSTP, albumin Myeloperoxidase	NA	Cys	sulfonamidesulfonamide	96–98
houltuynin	β -keto aldehyde	Hb, albumin	NA	Val, Lys	Schiff base, pyridinium	183
bergamottin	γ -ketoenal	Cyp3A4	NA	Gln	Michael adducts	184

^aDNCB: dinitrochlorobenzene. MRL-A: 1-{4-[(4-phenyl-5-trifluoromethyl-2-thienyl)methoxy]benzyl}azetidine-3-carboxylic acid. SMX: sulfame-thoxazole.

preparation in this way spreads the peptide mixture across two gradients, enhancing the sensitivity of detection.

2.2.3. Quantification. MS plays a major role in both relative and absolute quantification from complex sample mixtures. There are many potential applications for the use of this technology, notably for comparison of healthy volunteer vs diseased patient samples in identification of disease biomarkers.⁴⁶ It is widely understood that peak intensity alone is not a true indicator of the quantity of analytes due to the ionization efficiency and the detection of different peptides.⁴⁷ While this can be used to quantify similar peptides across different samples, moderated using standards throughout, more sensitive techniques have been developed in order to accurately quantify protein in mixed samples.⁴⁸

Stable isotope labels using tandem mass tagging (TMT) can be used for relative quantification, although this comes at a greater cost, and sample preparation is perhaps more time-consuming.⁴⁹ Isobaric tag for relative and absolute quantification (iTRAQ) is a method used to quantify multiple proteins in a single sample, using tags which covalently bind to the N-terminus and side chains of lysine amino acids.^{48,50} Individual samples are digested and tagged with one of eight different iTRAQ reagents.^{48,51} Each tag has the same molecular structure and overall mass; however, variable isotopic masses alter the m/z ratios detected.⁵² Samples are pooled and analyzed using LC-MS/MS, where database matching can identify the m/z of the reporter ion tags, and relative protein quantification can be calculated.^{53,54} While employing this technique is initially more time-consuming, the analysis of a single spectrum allows for a more streamlined approach.

Absolute quantification utilizes targeted MRM LC-MS/MS techniques. Here, labeled synthetic peptides of known concentrations are mixed within the samples. Synthetic peptides produce a standard curve which can be used to back calculate the concentrations of target peptides; unfortunately, synthetic standards are expensive and not always commercially available. As with relative quantification, iTRAQ is a well-defined reagent for this method.⁵¹ While data acquisition is relatively

straightforward, analysis requires powerful software to interpret the spectra. Multiple platforms exist consisting of databases enabling spectra to be matched with known proteins. However, many open access databases only contain peptide sequences rather than whole proteins. Commercial packages are available to enable full protein comparisons to be made, e.g., ProteinPilot; however, this is expensive software that requires significant information technology infrastructure.⁵⁰

While the use of TMT gives an accurate measurement of both relative and absolute protein quantification, its use in drug–protein adducts analysis is limited, especially with β -lactam antibiotics. β -Lactams covalently bind to the free amino group on lysine side chains, blocking the binding motif used by TMT, leading to partial labeling at best. Instead, radiometric methods are attributed as being the “gold-standard” method for the quantification of drug–protein adducts,^{55–57} which could potentially be used to quantify the covalent binding of β -lactam antibiotics to proteins *in vitro*. Proteins of interest, e.g., HSA, can be incubated with radiolabeled penicillins under the required conditions. Quantification of covalent bound drugs can be achieved using LSC after the unbound drug is washed away. As long as protein amount and radiation amount are known, the number of drug molecules per protein molecule can be calculated accurately.

2.3. Protein Adducts as Biomarkers for Exposure to Reactive Drug Metabolites. Bioactivation of drugs to reactive metabolites (RMs) that can covalently bind to macromolecules remains a major concern for drug development.⁵⁷ Drugs are usually metabolized via phase I and II reactions. Metabolism results in the formation of stable metabolites and loss of biological activity of the parent drug. This is known as detoxification. However, a large number of drugs have been shown to form RMs that could, in theory, provoke a toxic response. Therefore, high-throughput strategies have been put in place to screen for the formation of RMs in the early stages of drug discovery.⁵⁷ RMs, such as quinone imines, epoxides, and acyl glucuronides (Table 1), are reactive electrophiles, which can rapidly be inactivated by GSH conjugation or undergo covalent

Table 2. Drug Modified Albumin (Accession Number P02768) Detected in Patients^a

Amino acid	Peptide	BP	PIP	Amox	FLU	ABC	NVP	DCF AG	APAP
K12	FKDLGEENFK								
K137	KYLYEIAR								
K159	HPYFYAPELLFFAK								
K190	LDELRDEGKASSAK								
K195	ASSAKQR								
K199	LKCASLQK								
K212	AFKAWAVAR								
K351	LAKTYETTLK								
K432	NLGKVGSK								
K436	VGSKCCK								
K475	VTKCCTESLVNR								
K525	KQTALVELVK								
K541	ATKEQLK								
K545	EQLKAVMDFFAAVEK								
C34	ALVLIAFAQYLQQC PFEDHVK								
His146	RHPYFYAPELLFFAK								

^aBP, benzylpenicillin; PIP, piperacillin; Amox, amoxicillin; FLU, flucloxacillin; ABC, abacavir; NVP, nevirapine; DCF AG, diclofenac acyl glucuronide; APAP, acetaminophen.

binding to macromolecules. Therefore, drug–GSH conjugates or their further degradation products, i.e., cysteine or mercapturate products excreted in the urine, can be used as biomarkers for exposure to RMs. Furthermore, characterization of the structures of these thiol conjugates can inform the molecular mechanisms of drug bioactivation, thus leading to safer drug design.^{58,59} However, some RMs that have extremely short half-lives may bind directly to the enzymes responsible for their generation, leading to mechanism based enzyme inhibition (MBI). Another possibility is that the RMs may have great affinity toward intracellular proteins and react preferentially with these targets in different tissues. The intracellular proteins appear to be prominent targets for short-lived RMs or RMs that are likely to accumulate within cells. Furthermore, during chronic exposure, stable protein adducts are expected to accumulate in the body due to the long half-lives of proteins, e.g., 120 days for human Hb⁶⁰ and 21 days for HSA,^{32,36} and thus augment the sensitivity of detection of low level of RMs. Therefore, drug–protein adducts that reflect the systemic exposure to RMs *in vivo* may be more relevant when used as an alternative to GSH adducts for biomarkers. In this section, some common reactive drug (metabolites) and their reactions with proteins to form drug–protein adducts are discussed, with an emphasis on MS characterization of these adducts.

2.3.1. β -Lactam Antibiotics. β -Lactam antibiotics are widely prescribed for bacterial infections and are a frequent cause of

allergic reactions. β -Lactams are intrinsically reactive and can form drug–protein adducts with lysine residues on protein through the nucleophilic opening of the β -lactam ring. Recent studies investigating blood from patients using advanced MS methods have identified albumin as the major target for β -lactams. The precise haptenic structures and the exact location of modification on albumin have been defined for a number of β -lactams (Table 2).^{16,18,61} At low concentrations, different β -lactams appeared to selectively target different lysine residues in HSA, with noncovalent interactions positioning the drugs in favorable orientations in the protein-binding pocket to facilitate covalent binding. Thus, the three-dimensional shape of the drug, as well as its inherent chemical reactivity, determines the selectivity of covalent binding. However, at high concentrations and with prolonged incubation, multiple residues were modified by different β -lactams. Multiple sites of adduction are most likely observed *in vivo* with reactive drugs administered at high doses, e.g., penicillins; for the majority of RMs, more selective adduction is expected owing to the low levels of RMs. It is worth noting that multiple sites of adduction identified via tryptic digestion do not tell the number of adducts per molecule of proteins, which may be determined by analysis of whole proteins (top-down MS).

Flucloxacillin modifies up to 10 lysine residues in HSA *in vitro*, with Lys190 and Lys212 being the most reactive. Analysis of HSA isolated from the serum of eight flucloxacillin-tolerant patients

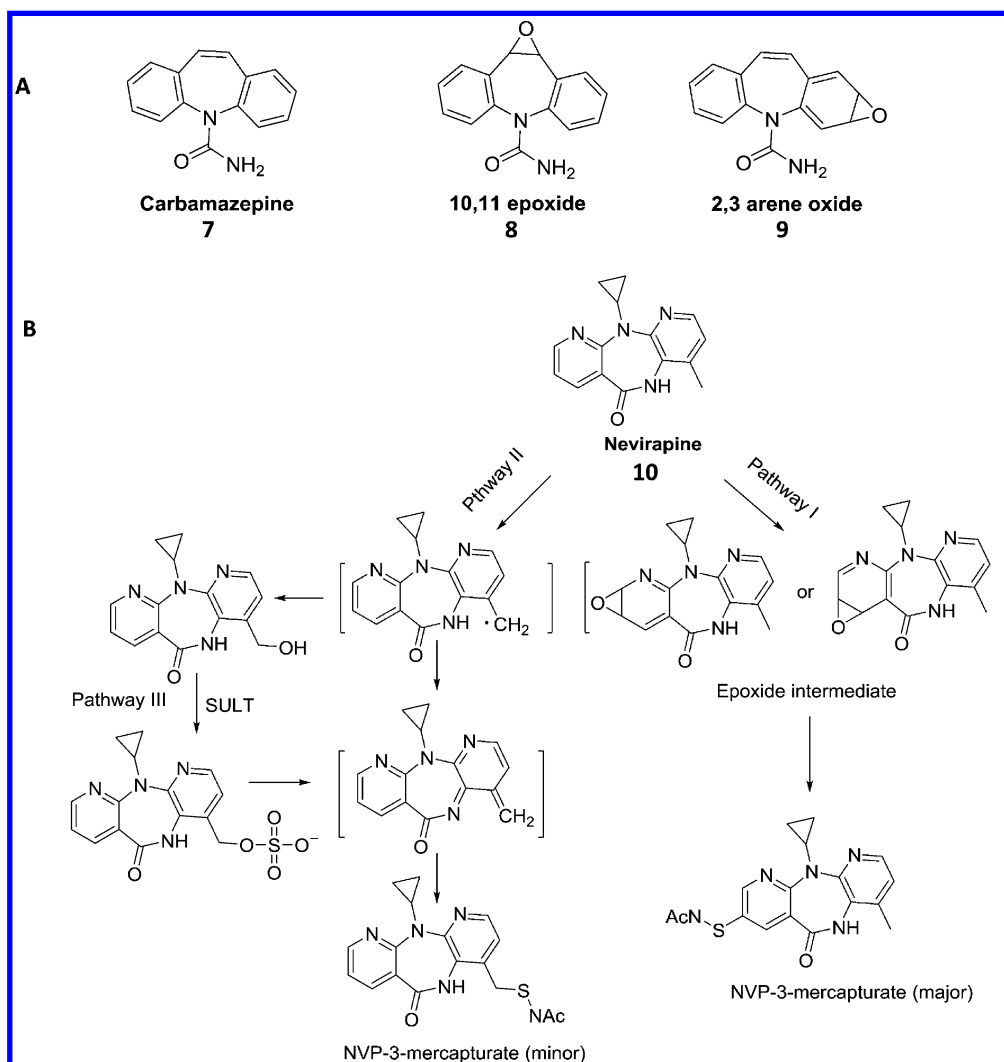


Figure 5. Epoxidation to generate reactive metabolites. Oxidation of carbamazepine by CYP enzymes leads to the formation of both arene oxides and epoxide metabolites (A); possible metabolic pathways for the formation of nevirapine reactive metabolites (B).

also showed that modification of Lys190 and Lys212 occurred in all the patients, with up to nine other modified lysine residues being detected. In addition, the detection of HSA adducts derived from its 5-hydroxymethyl metabolite provides the evidence of local hepatic exposure to this metabolite.¹⁸ Incubation of HSA with piperacillin results in the formation of two distinct haptens: a cyclized and a hydrolyzed form in which the dioxopiperazine ring had undergone hydrolysis.⁶¹ Piperacillin was able to modify up to 13 out of 59 lysine residues at high concentrations, with Lys541 being the only reactive residue at lower concentration. Four of these (Lys190, Lys195, Lys432, and Lys541) were also detected in plasma from 4 piperacillin treated, tolerant patients. In addition to the hydrolyzed and cyclized haptens of piperacillin, two further adducts derived from the desethyl metabolite of piperacillin were also detected in patients' plasma (Figure 4A). A typical MS/MS spectrum representing HSA peptide ⁵³⁹ATKEQLK⁵⁴⁵ modified by piperacillin at Lys541 is shown in Figure 4B. Compared with piperacillin, benzylpenicillin targeted different lysine residues at low concentrations: benzylpenicillin and its rearrangement product, benzylpenicillic acid, modified Lys199 and Lys525, respectively, whereas exposure to higher concentrations resulted in up to 16 further lysine modifications. Fourteen different modified lysine residues

were identified on HSA isolated from patients' serum (Table 2).¹⁶ Apart from albumin adducts, amoxicillin-modified transferrin and immunoglobulin were also detected when human serum was incubated with amoxicillin *in vitro*. The hapten was identified as amoxilloyl on Lys190, Lys199, and Lys541 with higher concentrations eliciting more extensive modification.⁶²

Unlike the penicillin class of β -lactams, meropenem and aztreonam contain a β -lactam ring fused to another ring that is different from the thiazolidine rings present in penicillins. Thus, Meropenem and aztreonam formed complex and structurally distinct haptenic structures with lysine residues on HSA. Each drug modified Lys190, with less common modifications at Lys12, Lys199, and Lys 351 for meropenem and Lys137, Lys432, and Lys541 for aztreonam.¹³

2.3.2. Arene Oxides and Epoxides. Bioactivation of aromatic compounds normally leads to the formation of arene oxides, while in the case of alkenes, the corresponding epoxides can be formed. Both arene oxides and epoxides are reactive and preferentially react with thiol nucleophiles on proteins. Carbamazepine is an example that generates an epoxide and a number of arene oxide reactive intermediates (Figure 5A). Carbamazepine–protein adducts have been detected in a number of *in vitro* systems. Incubation of CYP3A4 supersomes

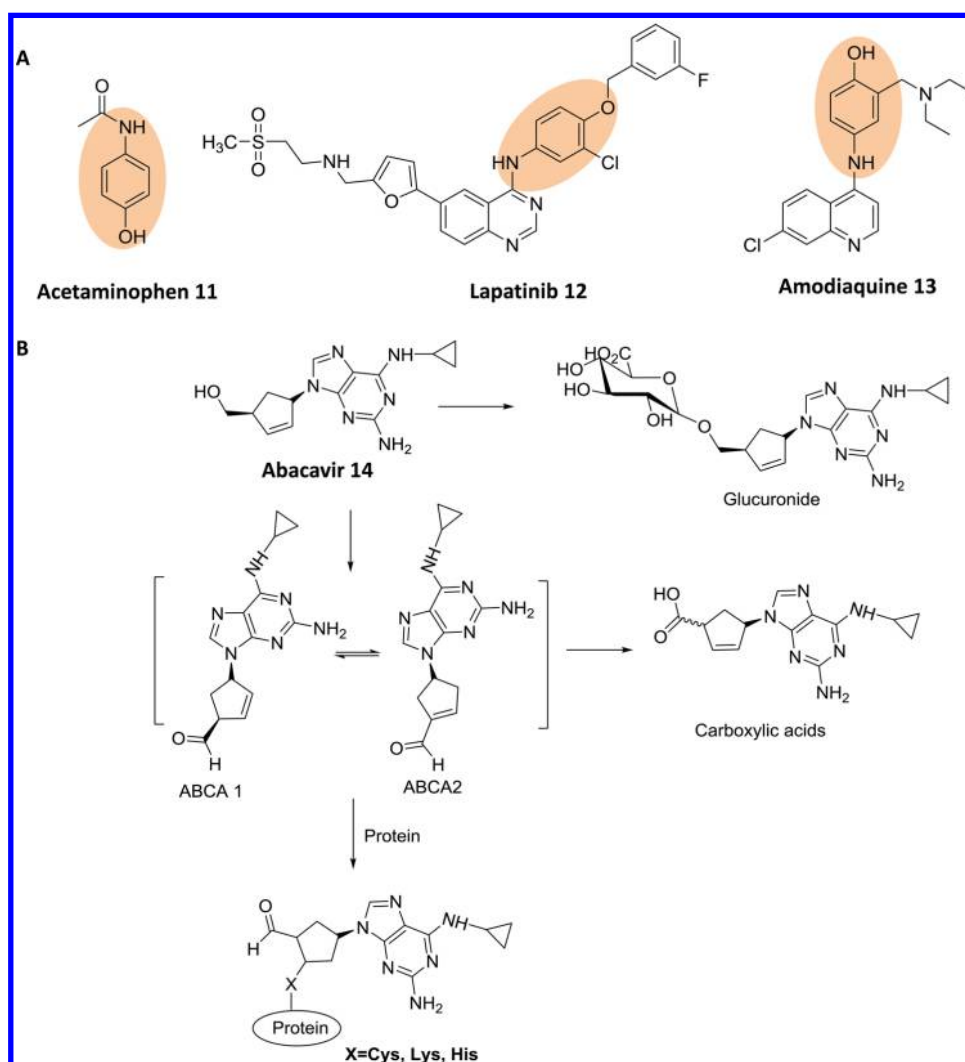


Figure 6. Michael acceptors as potential electrophiles. Drugs with aniline structural alerts can be oxidized to quinone imine reactive metabolites (A); possible metabolic pathways to form reactive α,β -unsaturated aldehyde metabolites that covalently bind to cysteine, lysine, and histidine residues on proteins (B).

with carbamazepine resulted in the formation of carbamazepine–CYP3A4 adducts, which may give rise to the autoantibodies detected in the sera of carbamazepine hypersensitivity patients.⁶³ Bioactivation of 3-hydroxy-carbamazepine and 4-hydroxy-phenytoin by myeloperoxidase led to covalent binding to the tyrosine of a lysozyme when used as a model protein.⁶⁴ Formation of hapten–protein complexes have also been detected with reactive metabolites derived from further oxidation of 2- and 3-hydroxy-carbamazepine, e.g., iminoquinone and O-quinone. Furthermore, carbamazepine 10,11-epoxide, an abundant and pharmacologically active metabolite believed to be chemically stable, has been demonstrated to be chemically reactive and form protein adducts with HSA and GSTP. MS analysis has identified the dihydrohydroxy thioether adduct at Cys47 on GSTP and two histidine adducts (His146 and His338) on HSA.⁶⁵

Nevirapine (NVP), a non-nucleoside HIV-1 reverse transcriptase inhibitor, appears to be predominantly metabolized by CYP3A4 and CYP2B6 to a number of hydroxyl metabolites in humans. Identification of two isomeric NVP mercapturates in the urine of patients and rats has provided the evidence that NVP undergoes bioactivation to heteroarene oxides and quinone methide intermediates *in vivo* (Figure 5B).^{66–68} The NVP 3-

mercapturate is likely to be derived from either 2,3-epoxide or 3,4-epoxide, whereas the NVP 12-mercapturate is a putative product of a quinone methide intermediate. The quinone methide intermediate can be derived from direct oxidation of NVP or from NVP-12-sulfate metabolite. It has been demonstrated that sulfation of 12-hydroxyl NVP followed by the dissociation of sulfate could also lead to the formation of the quinone methide. NVP-protein adducts have been detected in the skin, liver, and plasma *in vitro* and *in vivo* in animal studies^{69–71} using radiometric and immunochemical methods. However, neither the haptenic structure of these adducts nor the targeted amino acids were characterized by these studies. Therefore, it is impossible to determine which reactive intermediates are responsible for protein conjugation.

The *in vitro* incubation of 12-mesyloxy NVP, a chemical surrogate of 12-OH NVP, with model proteins such as HSA and Hb, has enabled the identification of 14 adducts involving cysteine, lysine, histidine, serine, tryptophan, and the N-terminal valine of Hb.⁶⁹ However, these findings do not accurately mirror the true adduct formed *in vivo* as 12-mesyloxy NVP is more reactive than 12-sulfoxy-NVP. Instead, NVP modification was only detected on cysteine and histidine when the 12-sulfate NVP metabolite was incubated with HSA and GSTP. Three histidine

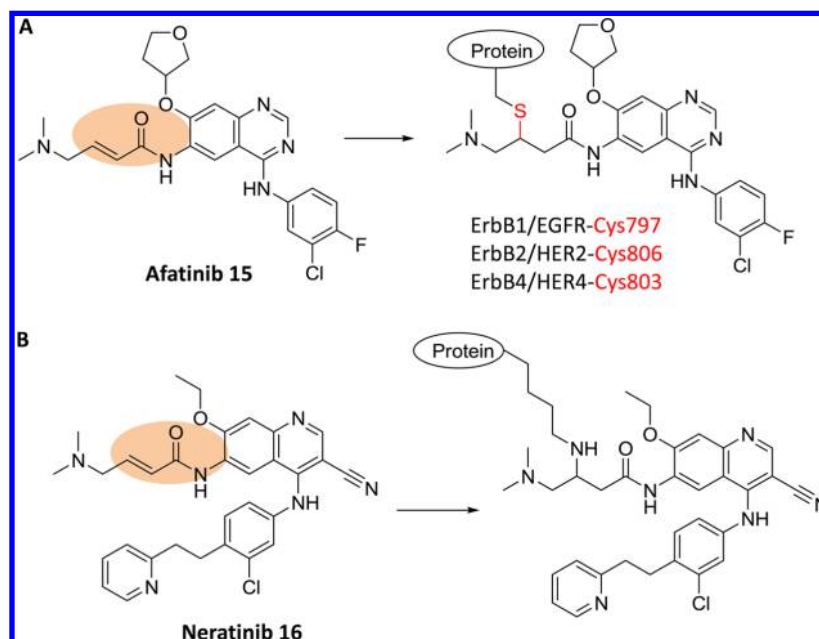


Figure 7. Covalent drugs containing weak Michael acceptors. Afatinib, a covalent inhibitor for the treatment of lung cancer, acts by covalent binding to cysteine residues at the catalytic sites of the receptors (A);⁸⁵ HSA has been identified as an off-target protein for some covalent inhibitors, for example, neratinib was found to bind to lysine residues on HSA (B).⁸⁶

residues (His146, His242, and His338), one cysteine residue in HSA, and a single cysteine residue in GSTP were identified. Nonetheless, NVP adducts formed with N-terminal valine in Hb⁶⁹ and His146 in albumin⁷² were detected in humans taking a therapeutic dose of NVP, reaffirming the relevance of *in vitro* studies.

2.3.3. Michael Acceptors. Michael acceptors, including quinones, quinone imines, quinone methides, and α,β -unsaturated aldehydes, are the most common types of RMs for numerous drugs. For example, in many drugs with aniline structural alerts, including acetaminophen (APAP), lapatinib, and amodiaquine (Figure 6A), bioactivation can lead to the formation of quinone imine intermediates; drugs with thiophene and furan moieties can undergo epoxidation, followed by ring opening to α,β -unsaturated aldehydes. In addition, Michael acceptors have been widely used as covalent drugs for cancer therapy such as the covalent kinase inhibitors ibrutinib and afatinib. Michael acceptors are normally considered as soft electrophiles and preferentially react with cysteine and histidine residues on proteins through 1,4-addition. In the case of α,β -unsaturated aldehydes, further cross-linking adducts may be formed after the initial 1,4 addition.

A classic example of the quinone imine formation is APAP, which is safe and effective at recommended doses, but can cause liver toxicity in overdose. APAP can be oxidized by P450 to the reactive *N*-acetyl-*p*-benzoquinoneimine (NAPQI), which can be detoxified by conjugation with GSH. However, overdose of APAP may result in covalent binding to proteins thus causing hepatotoxicity.⁷³ Covalent binding of NAPQI to cysteine residues in proteins has been detected in a number of *in vitro* and *in vivo* systems.^{74–77} Using an electrochemical flow-through cell coupled with LC-MS, Lohmann et al. was able to generate and detect APAP-modified Cys121 in β -lactoglobulin A.⁷⁸ More importantly, high concentrations of APAP adducts were detected in both adult and pediatric samples obtained from patients with acute liver failure (ALF). A recent pharmacokinetic study demonstrated that the level of APAP protein adducts in adults

with APAP overdose and ALF correlated well with the severity of toxicity, supporting their use as specific biomarkers for APAP toxicity in patients with ALF.^{76,79} However, the HPLC methods used in these studies to measure APAP–cysteine conjugates after proteolysis of all serum proteins do not define either protein targets or the position of cysteine residues within the proteins for modification. Therefore, an accurate quantitative relationship between APAP adducts at specific cysteine residues and APAP toxicity cannot be established.

Another important Michael acceptor is an α,β -unsaturated aldehyde, which can be generated by sequential metabolism of drugs with thiophene, furan, and β,γ -alcohol moieties. Abacavir (ABC), a nucleoside-analogue reverse transcriptase inhibitor, undergoes extensive oxidative metabolism in humans, yielding a number of isomeric carboxylic acids through a two-step oxidation process. Oxidation by alcohol dehydrogenase⁸⁰ followed by bond migration yields an electrophilic α,β -unsaturated aldehyde species (Figure 6B). Charneira et al. have shown that the ABC α,β -unsaturated aldehydes (ABCA) react with the N-terminal valine residue of Hb *in vitro*⁸¹ and in rats and patients.^{82,83} Recent *in vitro* studies using GSTP and HSA as model proteins by Meng et al. have demonstrated that ABCA can form novel intramolecular cross-linking adducts via Michael addition, followed by nucleophilic addition of the aldehyde with a neighboring protein nucleophile. Adducts were detected on Lys159, Lys190, His146, and Cys34 residues in subdomain IB of HSA. ABC-modified Cys47 and cross-linking adducts derived from Cys47 and Ser46 were identified in GSTP. More importantly, similar adducts were also detected in patients with ABC therapy.⁸⁴

In recent years, many covalent drugs that contain Michael acceptors have been developed for the treatment of cancer or hepatitis C infections.^{2,3} By selective covalent modification of a specific amino acid residues in the active site of the receptor or enzyme, this class of drugs provides increased biochemical potency and greater isoform selectivity, and can also overcome clinical resistance to mutant forms of the target enzymes.³ Afatinib and neratinib (Figure 7), which both contain an α,β -

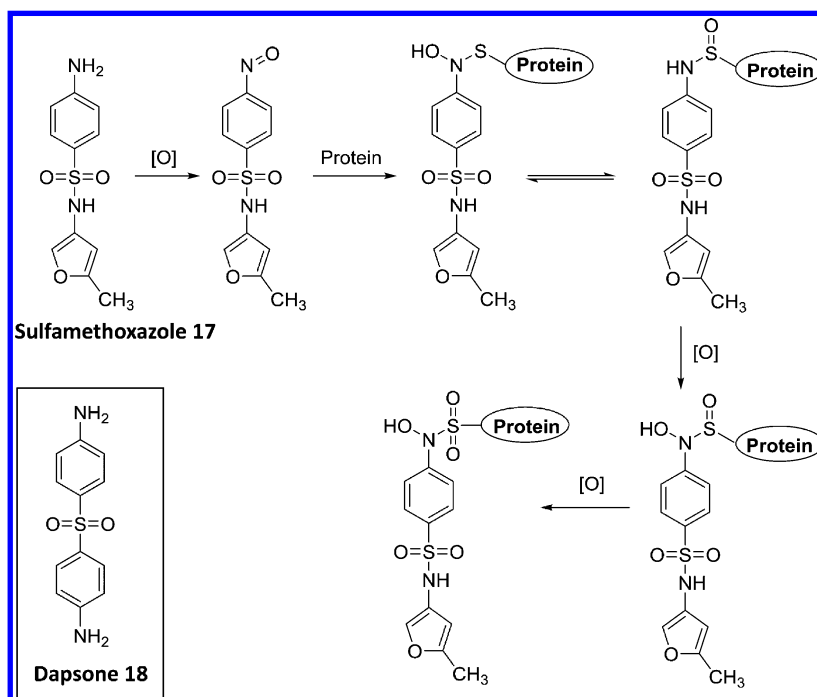


Figure 8. Oxidation of sulfamethoxazole to a nitroso reactive metabolite that forms a number of haptenic adducts with cysteine residues on proteins. Similar adducts may be also formed by dapsone with cysteine residues on proteins.

unsaturated acrylamide moiety, can covalently bind to cysteine residues in a number of tyrosine kinases and exhibit great clinical efficacy against tumors.⁸⁵ For example, afatinib is capable of forming Michael adducts with cysteine residues within the catalytic sites of EGFR (Cys797), HER2 (Cys805), and HER4 (Cys803) (Figure 7A). Despite promising efficacy across a broad range of disease, there have been great concerns that covalent drugs might cause potential off-target toxicity. However, drugs designed to bind covalently to specific pharmacological targets generally exhibit a low degree of interaction with off-target cellular proteins owing to the weakly electrophilic warheads that are incorporated into them and a much greater affinity for the target receptors or enzymes. The off-target reactivity of covalent drugs has been assessed in a quantitative and qualitative manner by using ¹⁴C-labeled compounds. Neratinib has been shown to form albumin adducts via Michael addition with lysine attacking the β -carbon of the amide moiety. MS analysis of neratinib-modified albumin confirmed Lys190 of HSA was covalently bound to neratinib (Figure 7B).⁸⁶ Further quantitative analysis has shown that covalent binding of neratinib to HSA is pH, time, and temperature dependent but not substrate concentration dependent, especially in the therapeutic range.²⁴ Apart from albumin, identification of other critical proteins potentially targeted by covalent drugs is necessary for the assessment of the toxicity associated with them. For example, the enzyme ferrochelatase that catalyzes the conversion of protoporphyrin IX into heme has been recently found to be an off-target of many kinase inhibitors, providing a possible explanation for the phototoxicity associated with these drugs in melanoma patients.⁸⁷

Despite the unique advantages of drugs which act as “covalent inhibitors”, there are general concerns regarding immune-mediated toxicity that could arise from covalent binding with off-target proteins. Several approaches, including increase of the inherent affinity to target enzymes to improve the selectivity and the use of weak electrophilic functionalities to minimize the

nonspecific reactivity, have been employed for designing and optimizing this class of drug. As a new emerging class of therapy, little is known of their safety profiles. Several cutaneous adverse reactions associated with “covalent inhibitors” have been reported in clinical trials;^{88,89} however, the underlying mechanisms are not clear. Advanced MS-based proteomics that enable us to identify the off-target proteins, coupled with *in vitro* model systems for the elucidation of possible immunological events, may provide insights into both the efficacy and safety of this class of drug.

2.3.4. Aromatic Amines. Compounds containing aromatic amines are well-known to induce a variety of toxicities including carcinogenicity and hypersensitivity. The primary bioactivation pathway of an aromatic amine is the oxidation of the amino group to form a hydroxylamine, which can form arylnitroso derivatives by further oxidation or other reactive species by sulfation/acetylation.⁹⁰ The arylhydroxylamines and their oxidized arylnitroso derivatives can react with cysteine residues of GSH or proteins to form sulfenamide and sulfinamide adducts, respectively. In addition, direct reactions of hydroxylamines with other amino acid residues, including histidine and tryptophan, have also been reported.⁹¹ Both Hb and HSA have been targeted by a number of aromatic amines *in vitro* and in animals.

Sulfamethoxazole (SMX) is a sulphonamide containing antimicrobial associated with a high incidence of hypersensitivity including fever, rash, and hepatitis. The hypersensitivity reactions are thought to be a consequence of bioactivation to the hydroxylamine metabolite (SMX-NOH) and its further oxidation product, nitroso-sulfamethoxazole (SMX-NO). These reactive metabolites act as haptens and activate T-cells via the covalent modification of MHC bound peptides and non-MHC associated proteins.^{92–94} SMX-NO binds extensively to cysteine residues on serum and cellular proteins, forming complex and multiple haptenic structures including sulfonamides, *N*-hydroxysulfonamides, and *N*-hydroxysulfonamides

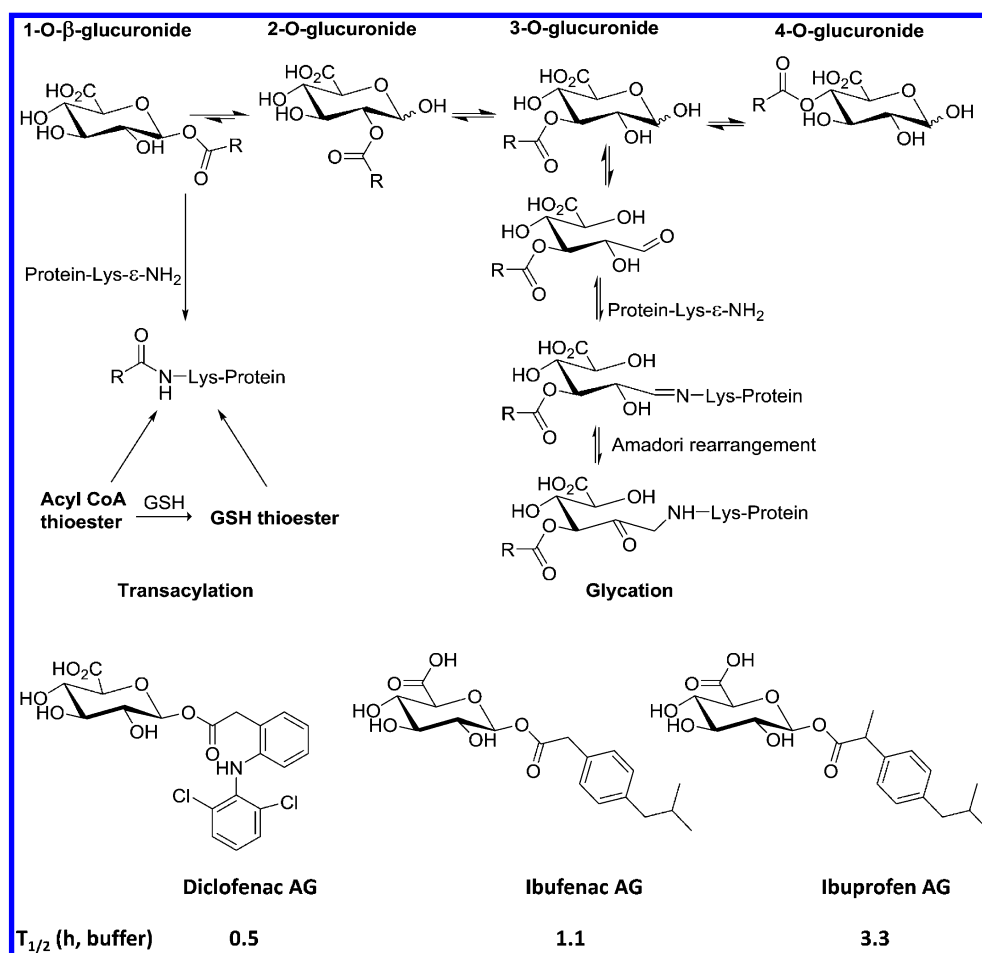


Figure 9. Mechanisms of protein adduct formation by reactive acyl glucuronide and thioester metabolites of carboxylic acid containing drugs.

(Figure 8).^{95–97} Likewise, dapsone is oxidized to a number of RMs, leading to protein adduct formation. Dapsone–protein adducts have been detected in dendritic cells as well as keratinocytes.^{98,99}

2.3.5. Acyl Glucuronides. Many carboxylic acid-containing drugs such as nonsteroidal anti-inflammatory drugs (NSAIDs) are metabolized to β -1-*O*-acyl glucuronides (AGs), which are able to form adducts with nucleophiles on proteins. In theory, the resulting protein adducts may cause toxicity through the impairment of physiological functions. AGs can modify proteins through two distinct pathways. Direct reactions with certain amino acid residues on proteins, such as lysine, cysteine, and arginine, lead to transacylation adducts. Alternatively, migration of the acyl group may yield the 2-, 3-, and 4-acyl isomers, which are relatively less labile to direct hydrolysis but exhibit great chemical reactivity of anomeric sugar esters. Reaction with amine nucleophiles followed by an Amadori rearrangement generates a more chemically stable 1-amino-2-keto product (Figure 9).¹⁰⁰ Structure–reactivity studies have revealed that carboxylic acids with a higher degree of alkyl substitution at the α -carbon exhibit increased stability;¹⁰¹ thus, the ibuprofen AGs display a longer half-life (3.3 h in pH 7.4 buffer) than AGs of diclofenac and ibufenac.¹⁰⁰

HSA has been identified as a major target for many AGs in plasma because of its abundance and the presence of several accessible nucleophilic amino acid side chains.^{102–105} However, other proteins in the liver, kidney, skeletal muscle, and intestine have also been reported as targets for AGs.¹⁰⁶ For example, the

AG of diclofenac and many other carboxylic acid containing NSAIDs has been found to selectively bind to several proteins located on the apical (bile canalicular) domain of the hepatocyte plasma membrane.¹⁰⁷ By use of immunochemical techniques, hepatic dipeptidyl peptidase IV has been identified as a common target for the AGs of a number of drugs including zomepirac, although other targets are yet to be found.

In addition to AGs, carboxylic acid drugs can undergo bioactivation to form electrophilic acyl-coenzyme A thioester derivatives (acyl-CoAs), which have been shown to be up to 40–70 times more reactive.¹⁰⁸ Acyl-CoA derivatives of several carboxylic acid drugs including zomepirac and tolmetin have been detected *in vitro* and *in vivo*.^{109,110} *In vitro* incubation of the CoA conjugates of a number of NSAIDs with human liver microsomal proteins confirmed that the reactivity of CoA derivatives was significantly higher compared to those of their corresponding oxidative metabolites and AGs. Therefore, it is plausible that CoA conjugates can contribute to the toxicity observed with carboxylic acid drugs.^{111–113}

Despite extensive study of the chemical reactivity of AGs, their precise role in toxicity remains undefined. Retrospective analysis of the toxicity associated with numerous carboxylic acid containing drugs revealed that the stability of AGs exhibits some degree of correlation with the risk of toxicity. Therefore, the half-lives of AGs have been widely used for selection of compounds in drug development. However, this has been challenged based upon the poor correlation between the extent of covalent binding and the risk of toxicity for certain NSAIDs,

such as ibuprofen¹¹⁴ and naproxen.¹¹⁵ Therefore, other factors such as daily dose, oxidative stress, and transporters, along with chemical reactivity, might be required for the holistic assessment of AG toxicity risks.

3. FUNCTIONAL ASPECTS

3.1. Understanding the Function of Drug–Protein Adducts. While the detection and discovery of drug–protein adducts has been difficult, new MS techniques have made this process more feasible, leading to the detection and precise structural definition of a number of adducts. Understanding the mechanistic function of these adducts, however, remains a challenge. Although we have discussed the resurgence of drugs which bind covalently as part of their pharmacological function, most drugs in use today bind to receptors within the body in a noncovalent manner and are usually designed to limit binding to proteins.² Unfortunately, the metabolic systems in the liver that can cause the formation of RMs make drug–protein adduct formation inevitable. Adduct formation seems to correlate with the incidence of ADRs, but it has been difficult to make a mechanistic link between the two. The nature of ADRs is highly variable; they are unrelated to the normal pharmacological action of the drug and are often categorized as idiosyncratic ADRs.¹¹⁶ As ADRs only occur in some patients, these reactions can be attributed to the individual biology of patients suggesting that highly specific mechanisms are involved. However, the exact fate of drug–protein adducts and their roles in ADRs remain unclear.

Many studies have addressed the role of adduct formation in drug toxicity that can be categorized into two major pathways: direct toxicity and immune activation. In direct toxicity, either the adduct itself is toxic to the body or the drug binds to a protein which has a functional role in the body. In the latter, the drug binds in such a way that it changes the biological function of that protein. In the immune activation pathway, an adduct can be recognized by the body's immune system as a foreign antigen. This can cause an adverse immunologically driven event. The nature of these two pathways will be discussed with reference to specific examples, and a prominent feature of this analysis will show that even within one pathway a vast array of mechanisms appear to be involved.

3.2. Direct Toxicity. The structure of a protein is highly specific; therefore, covalent binding will alter the structure of a protein and affect its function.⁵⁵ Incidentally, many drugs act on liver proteins causing hepatotoxicity.^{117–119} Although other organ systems are also affected by adduct formation, the liver seems to have a functional role in mediating these reactions. The formation of RMs and their direct impact on the liver including the generation of reactive oxidative species and covalent binding to crucial proteins can be harmful, but RMs are not usually generated in sufficient quantities to bind to proteins; they can bind to thiols such as glutathione and will be excreted as chemically inert compounds.¹²⁰ There are a number of such protective mechanisms in the liver such as the NRF2/KEAP pathway.¹²¹ However, when these protective mechanisms are depleted, drug metabolites that can bind to functional proteins within the liver may induce direct toxicity.¹²² The previous section outlines examples of some of these target proteins. Importantly, the focus for this review is the contribution of drug–protein adducts to toxicity rather than the action of RMs and their depletion of protective mechanisms. Drugs can bind to proteins to adversely alter protein function, also known as mechanism based inhibition. Alternatively, a protein adduct can

itself be toxic. A few examples have been chosen to represent each of these mechanisms.

3.2.1. Liver Toxicity. The painkiller, APAP, can cause acute hepatotoxicity in drug overdose.¹²³ As previously discussed, APAP is biotransformed during phase I metabolism into its reactive metabolite NAPQI.¹²⁴ Early studies identified that at nontoxic doses of APAP, NAPQI can be detoxified by conjugation with glutathione to be excreted from the body.¹²⁵ The detoxification mechanism is further evidenced by the treatment with *N*-acetyl cysteine in overdosed patients to decrease the effects of hepatotoxicity.¹²⁶ Additionally, a correlation was made between APAP–protein adducts and the incidence of toxicity in mice. Using an immunohistochemical approach, hepatic necrosis was found to correlate to a greater presence of APAP adducts; it was deduced that toxicity was caused by the binding of NAPQI to crucial proteins in the liver.¹²⁷ Collectively, these binding studies in APAP have provided a framework to study other quinoneimines which interact with nucleophilic residues on cellular macromolecules.¹²⁸ Like APAP, the antimalarial drug amodiaquine, also forms a quinonimine metabolite, which binds to crucial liver proteins and causes liver necrosis.¹²⁹ The RMs formed can have a number of adverse effects. For example, significant lipid peroxidation takes place in amodiaquine toxicity caused by free radicals generated from the original RMs.¹³⁰ However, RM-modified proteins also have their own functional role in toxicity, for example, amodiaquine adducts can activate an immune response.¹³¹ A recent study in PD-1 (programmed cell death protein 1) –/– mice demonstrated T-cell mediated reactions upon treatment with amodiaquine.¹³² Another study has also shown that even APAP has the potential to drive an adaptive immune response.¹³³ It is becoming more apparent that drug–protein adducts have a functional role in immune activation.¹³⁴

3.2.2. Mechanism Based Inhibition. MBI is a form of enzyme inhibition which occurs when a RM binds to an enzyme. MBI can be reversible, where RMs form a tight noncovalent bond through coordination to the heme prosthetic group of the enzyme. In contrast, irreversible inhibition involves covalent binding of RMs with amino acid residues at the active site of the enzyme. Covalent modification of the enzyme can lead to antigen formation and in some cases induce an autoimmune response. The classical example of MBI is tienilic acid which was withdrawn from the market due to its hepatotoxic effects. Tienilic acid was released in the 1970s as a uricosuric diuretic drug. It is bioactivated by CYP2C9; however, one of the reactive species generated upon bioactivation also acts as a “suicide substrate” and alkylates CYP2C9, thus inhibiting its function.¹²⁰ One study investigated tienilic acid with a closely related isomer to demonstrate that the isomer can cause covalent binding to various microsomal proteins but that only tienilic acid binds CYP2C9 in a very specific manner.¹³⁵ Antibodies against tienilic acid have been detected which may be responsible for the autoimmune responses.^{136,137} Lapatinib, a dual tyrosine kinase inhibitor used for the treatment of breast cancer, is a more recent example of a drug which acts as a mechanism based inhibitor of CYP3A4. It is possible that lapatinib causes MBI of CYP3A4 via covalent binding of a reactive quinoneimine metabolite to the enzyme. Interestingly, Takakusa et al. demonstrated that MBI of CYP3A4 by lapatinib was mainly due to the formation of quasi-irreversible complex formed by a reactive nitroso intermediate and the enzyme.¹³⁸ Lapatinib is a particularly interesting example to conclude this section because it is an MBI of CYP3A4, but it has the ability to form a quinoneimine which could lead to direct

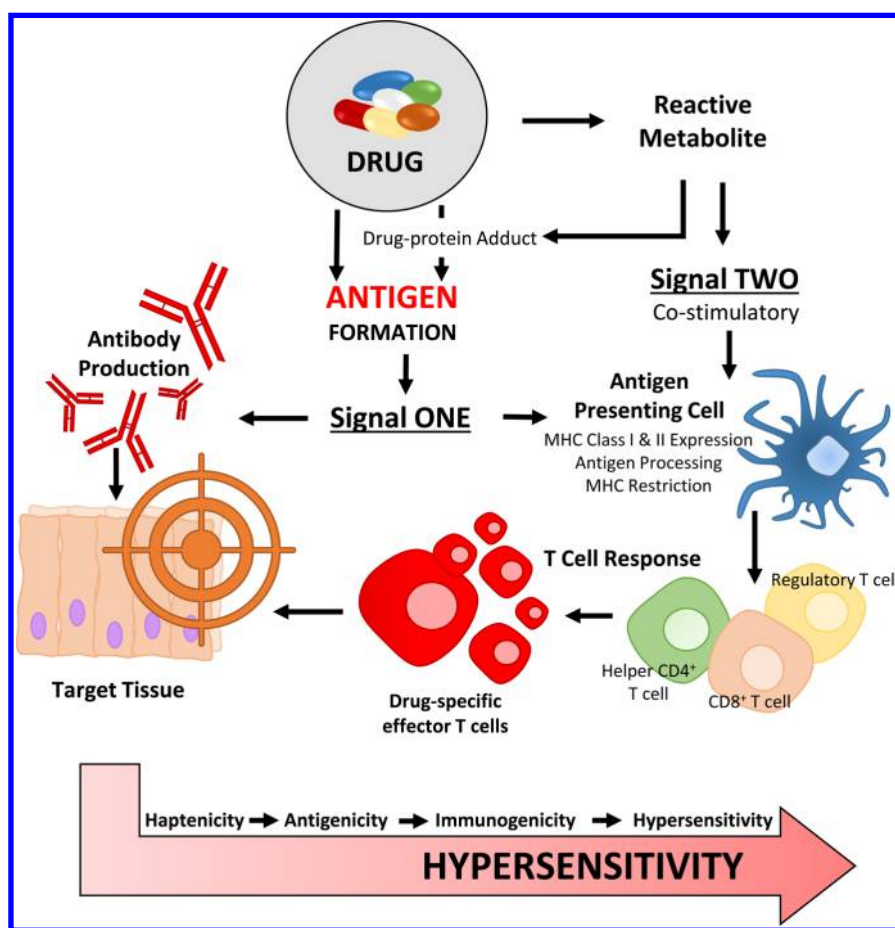


Figure 10. Hapten model of drug hypersensitivity. Haptens are small molecules which can bind to proteins to become antigenic. These adducts are recognized by the body's immune system as foreign and are therefore taken up by antigen presenting cells. The conjugated protein is processed, and peptide fragments are presented upon MHC. Self-peptides will not activate the immune system. It is understood that drug modified peptides presented on MHC are responsible for activating T-cells in order to provoke an immune response. These T-cells can help to activate B-cells to release adduct specific IgE, or they can become drug specific effector T-cells to initiate an adaptive immune response. Antigen recognition alone (signal one) is not enough to drive an immune event; an environment of cell stress and danger signaling (signal two) which can initiate the required costimulatory pathways may be required to trigger an immune response.

toxicity. This toxicity has recently been associated with HLA DRB1*07:01, which suggests the involvement of the immune system.^{139,140} Though different mechanisms are present within drug toxicity, they seem to be linked as a chain of events. What is becoming clearer is that the final event in this chain is often the activation of an immune response.

3.3. Immunological Activation. Immune responses to drug–protein adducts manifest as both immediate IgE mediated reactions and delayed type T-cell mediated reactions which are clinically distinct (Figure 10). Various clinical outcomes may occur with immediate reactions targeting the skin to cause rashes and maculopapular exanthema and delayed reactions causing more severe symptoms such as Stevens Johnson's syndrome and toxic epidermal necrolysis.¹⁴¹ In addition, the delayed nature of idiosyncratic drug induced liver injury may also be attributed to an underlying immunological mechanism.¹⁴²

3.3.1. Immediate Hypersensitivity Reactions. Immediate reactions are caused by the recognition of a drug or drug–protein adducts by IgE antibodies. The formation of antigen specific IgE is a B-cell mediated T-cell interaction. Upon initial exposure, a drug modified peptide is presented to T-cells and B-cells. T-cells provide stimulatory signals to B-cells which release antigen specific IgE. The cross-linking of IgE initiates the release of inflammatory mediators such as histamine, prostaglandins, and

cytokines. These inflammatory mediators establish a milieu of cell stress resulting in the clinical eventualities described.¹⁴¹

Drug specific antibodies have been detected for a number of drugs including penicillins, cephalosporins, and sulfamethoxazole.^{143–145} Studies on immediate hypersensitivity to amoxicillin have demonstrated the presence of highly specific IgE antibodies which can recognize amoxicillin but not the structurally similar compound benzylpenicillin.¹⁴⁶ A number of similar studies have taken place with other penicillins, which corroborate the importance of the penicillin side chain in determining the specificity of these reactions.¹⁴³ While the clinical manifestations of immediate and delayed type reactions (DTRs) differ, the mechanistic presentation of a drug modified peptide to a T-cell is a shared molecular initiating event. Therefore, drug–protein adducts play a central role in the mechanism of drug induced hypersensitivity, making the study of these adducts a focal point for research in this field.

3.3.2. Mechanism of Delayed Type Hypersensitivity. Many models for DTRs were first studied by Landsteiner and Jacobs when investigating the role of simple chemical compounds in the sensitization of guinea pigs in allergic contact dermatitis.¹⁴⁷ DTRs generally involve T-cell mediated adaptive immune responses. However, additional danger signals mediated by the innate immune system are essential for this to take place.¹⁴⁸

Drug–protein adducts play an important role in both innate and adaptive immunity, and these interactions have been studied extensively.¹⁴⁹

3.3.3. Role of the Innate Immune System in Drug Hypersensitivity. The innate immune system bridges the deleterious effects of direct RMs toxicity to the initiation of an immune response. Direct toxicity causes cell death, which initiates danger signaling. The release of damage associated molecular patterns (DAMPs) can activate pattern recognition receptors such as toll-like receptors (TLRs) and nucleotide-binding oligomerization domain-like receptors (NLRs), leading to upregulation of pro-inflammatory cytokine and chemokine production.¹⁵⁰ HMGB1, which is one such DAMP, has shown to activate TLR2, -4, and -9 and is recognized as an early biomarker for drug induced liver injury.¹⁵⁰ NALP3 is an NLR which forms an inflammasome upon activation. Inflammasomes activate caspases which cleave pro-inflammatory cytokines to their active form.¹⁵¹ Upon cell death in APAP induced liver injury, free DNA released can activate TLR9 and interact with the NALP3 inflammasome to contribute to hepatotoxicity.¹⁵² While the pathways described are all downstream effects of direct toxicity, it is only in an environment of “danger signaling” in which the maturation of dendritic cells (DCs) can occur. The maturation of DCs is one of the key factors required to initiate an adaptive immune response.

A number of studies have investigated the effects of protein modification on the innate immune system, mainly pertaining to contact allergens. Metal ions are common contact allergens; in particular, nickel ions (Ni^{2+}) have been shown to interact covalently with histidine residues on TLR4. Ni^{2+} activates human TLR4 but not mouse TLR4 via covalent binding of Ni^{2+} to histidine residues that caused the dimerization of TLR4.¹⁵³ Additionally, electrophiles such as DNCB and NAPQI can activate nrf2 through covalent binding to cysteine residues within the Keap1 protein.^{154,155} A better understanding of covalent binding in innate immunity may provide therapeutic targets to reduce the severity of drug hypersensitivity reactions.

3.3.4. Role of Adaptive Immunity in Drug Hypersensitivity. Although multiple models of drug hypersensitivity exist, which include a number of theories surrounding noncovalent interactions between drugs and immune receptors, the hapten mechanism requires a drug to covalently modify a protein for antigen recognition. In order to prime naïve T-cells, DCs matured under the influence of danger signals are thought to engulf a drug–protein adduct and present a drug modified peptide to a naïve T-cell resulting in drug-specificity. This forms the basis for a T-cell mediated, adaptive immune response and this interaction has been widely investigated.

β -Lactam antibiotics covalently bind to lysine residues on the surface of proteins.⁵ Hans Ulrich Weltzein studied the application of the hapten model in relation to β -lactam hypersensitivity extensively. It has been shown that a portion of the immunogenic epitope can be recognized by the T-cell.¹⁵⁶ Synthetic peptides were used to explore the role of drug location on a peptide sequence and their ability to induce an immune response. This *in vitro* study demonstrated that penicillin-modified peptides were responsible for an immune response in humans.¹⁵⁷ Another study altered the basic structure of penicillin to remove the thiazolidine ring and the side chain deriving two molecules: cefaclor and 6-aminopenicillanic acid. When tested for immunogenicity neither molecule exhibited a response in comparison to the unaltered drugs.¹⁵⁸ This illustrated that the precise structures of epitopes are important for T cell

recognition; hence, it is essential to characterize the exact nature of antigen presented to T cells. SMX has been used as a model drug for a number of early studies to support the hapten hypothesis. Multiple mechanisms including both a covalent and noncovalent interaction have been shown to activate immune responses.⁹² As we have discussed, SMX can form a number of reactive metabolites including SMX-NHOH and SMX-NO. Using flow cytometry, one study successfully stained these metabolites being presented on the surface of neutrophils.¹⁵⁹ Additionally, an increase of SMX-NO adducts have been demonstrated under conditions of danger signaling.¹⁶⁰ SMX has also been shown to have a genetic link with the HLA DQ gene family; this has been confirmed by the presence of HLA DQ restricted CD4+ lymphocytes.¹⁶¹

Flucloxacillin induced liver injury has been shown to be associated with HLA B*57:01.¹⁶² A flucloxacillin adduct may be responsible for activating a T-cell mediated response in the liver. This has been confirmed by the presence of drug specific T-cells in patients.¹⁶³ Mainly CD8+ T-cells expressing liver homing receptors CCR4 and CCR9 were observed suggesting that these T-cells could have the cytotoxic potential to destroy liver cells. Flucloxacillin-specific T-cells were restricted by HLA B*57:01 and required antigen processing to trigger a T-cell response. Antigen processing is determined using a pulsing assay. Here, antigen presenting cells are incubated with a drug for a short period of time before the drug is washed out. T-cell assays are performed to specifically investigate the uptake of a drug antigen and its presentation on a T-cell thus testing the hapten model. Another study compared patient and volunteer clones to illustrate that patients' clones responded to flucloxacillin protein adducts, requiring antigen processing, whereas volunteer clones could be stimulated by the drug directly without antigen processing.¹⁶⁴ Flucloxacillin-specific T cells generated *in vitro* from healthy volunteers carrying HLA B*57:01 were shown to be cytotoxic in the presence of liver cell lines transfected with HLA B*57:01.¹⁶⁵ Although these studies address the functional aspects of T-cells and their recognition of the flucloxacillin molecule, the exact antigens responsible for activating drug-specific T-cells still has not been identified. Some animal models have identified flucloxacillin protein adducts in the liver,¹²² but no study has yet taken flucloxacillin protein adducts generated *in vitro* and shown a T-cell response.

On the contrary, studies in piperacillin have shown some success in relating drug–protein adducts to their immunological roles. Piperacillin hypersensitivity is common in patients with cystic fibrosis. A number of different piperacillin albumin adducts have been identified in patient plasma. These modifications have been replicated and characterized in cell culture medium. Piperacillin specific T-cell clones are also activated upon pulsing Epstein–Barr virus transformed B cells (EBVs) with piperacillin. Most importantly, synthetic piperacillin albumin conjugates have been shown to activate piperacillin specific T-cells *in vitro*. To the best of our knowledge, this is the only study which has demonstrated the activation of a T-cell by a drug–protein adduct.⁶¹ Interestingly, another study replicated these results with the addition of flucloxacillin albumin conjugates to demonstrate that HSA bound to both flucloxacillin and piperacillin decreased T-cell proliferation against HSA bound solely to piperacillin. Flucloxacillin-HSA conjugates did not cross-react with piperacillin specific T-cells.¹⁹

NVP can cause skin and liver injury with a number of different genetic associations.¹⁶⁶ It seems to be the case that the skin and liver injury occur via different pathways entirely. Sharma et al.

successfully characterized the binding of NVP metabolites to human and rat skin *in vitro*. A skin rash similar to that occurs in humans was found to correlate to the covalent binding of NVP to keratinocytes in a rat model.⁷⁰ A major outcome of this study was that the site of adduct formation occurred close to the reaction location. However, an attempt to model NVP induced liver injury in the same manner showed the presence of extensive NVP adducts on hepatic tissues, but there was no evidence of liver injury in the rats. They postulated that liver injury may be caused due to the direct formation of a quinone methide RM.¹⁶⁷ Therefore, different clinical manifestations of a drug may be mediated by entirely different mechanisms. More recently, in a mouse model, they have shown that NVP can produce significant liver injury when PD-1 was blocked, indicating immune tolerance plays an important role in NVP induced liver injury.¹⁶⁸

3.3.5. Advances in Understanding Immune-Mediated Adverse Reactions. Although the idiosyncratic nature of these reactions has been attributed to genetic variability among patients,¹⁶⁹ challenges still focus around identifying the chemical nature of the antigenic determinants which are responsible for these reactions. An understanding on how the field of immunology has progressed in recent years and advances in the sensitivity of technology available to us can help deduce whether the hapten model is still current within idiosyncratic drug reactions.

Much progress has been made on understanding the immunological mechanisms of adverse reactions since the initial hapten hypothesis was postulated. In particular, recent genome wide association studies have demonstrated that genetic variability plays a key role in individual susceptibility. A number of genetic associations have been found with drug induced hypersensitivity reactions and many of these with HLA alleles.¹⁷⁰ Although HLA alleles have shown to be linked with drug hypersensitivity reactions, not every patient who is a carrier of that allele will have an immune response. Co-stimulatory pathways are involved in eliciting an immune response, and the priming of naive T-cells to drugs has shown to be more effective when some of these pathways are blocked.¹⁷¹ It is evident that genetic associations will be a key to understanding the individual susceptibility; however, other factors including the chemical nature and the level of antigen and the phenotype of the cell will certainly have great impact on the severity of hypersensitivity.

4. DISCUSSION

Major advances in proteomics technology including two-dimensional SDS–PAGE separation techniques combined with MS and/or immunoblotting techniques have allowed for a more precise identification of protein targets and the exact location of adducts.⁵⁷ A large number of target proteins for drugs have been identified, and a Web-accessible Target Protein Database is now available for the storage of such information and to facilitate the identification of common protein modification patterns of different drugs.¹⁷² In addition, quantitative analysis of drug–protein adducts formed *in vivo* employing new proteomic technologies is critical to unveiling the links between covalent binding and organ toxicity. However, the progress in identifying the functional roles of these drug–protein adducts has been slowed by a number of different challenges within the field.

First, a large number of drugs associated with hypersensitivity have been shown to form reactive metabolites that can modify proteins both *in vitro* and *in vivo*, but the “reactive metabolite theory of drug hypersensitivity” is still based primarily on a “global association” between *in vitro* bioactivation and clinical

outcome. Because of a lack of data on absolute levels of *in vivo* covalent binding that could lead to a toxic outcome, it is almost impossible to determine whether the parent drug, stable metabolite, or RMs are involved, and which protein targets are critical for toxicity. Hapten density may also be an important aspect in immunogenicity. Often, idiosyncratic drug reactions are misidentified as being unrelated to the administered dose. However, dosage can have a role in determining the clinical outcome of a reaction. For this reason, the quantification of drug–protein adducts is essential. Additionally, there may be a certain threshold for toxicity which can be identified retrospectively. The main issue is that at present, the technology to investigate T-cell responses to short-lived chemically reactive intermediates does not exist.¹⁷³ Without this knowledge, it is currently impossible to predict the potential for a given compound that causes idiosyncratic toxicity via bioactivation.

Second, the nature of the antigenic determinants that trigger an immunological response to drug–protein adducts remains to be defined. The most significant study in drug hypersensitivity in recent years has been unravelling the mechanism behind the association between abacavir and HLA B*57:01.^{149,174} In this study, peptides were eluted from drug treated EBV transformed B cell lines, and the peptide repertoire was shown to change in the presence of the drug. Early studies which investigated the nature of drug modified peptides presented on MHC have yet to be replicated, and there has been an absence of studies which introduces a synthetic drug modified protein or peptide to activate drug specific T-cell clones. As yet, this has only recently been done with piperacillin modified albumin. The exact nature of the drug–peptides which are presented have yet to be elucidated. Another major challenge has been identifying the mechanistic role of genetic associations. While flucloxacillin has shown HLA restricted responses *in vitro*, the preferential role of these HLAs is still unknown for most drugs. Aside from abacavir, the question has yet to be answered as to whether HLA's have a functional role in drug hypersensitivity reactions. Interestingly, the pathway required for the release of IgE and also for an adaptive immune response both require the formation of a drug–protein adduct. Therefore, if the same signal 1 pathway manifests as different clinical phenotypes then there is a possibility that the signal 2 pathway which is adduct independent may be the determining factor in drug hypersensitivity reactions. This further supports the importance of inter individual variation in patients.

Accumulative evidence has shown that drug–protein conjugation has played a role in the pathogenesis of serious adverse drug reactions, but whether it plays a predominant or an accessory role will depend on multiple factors. It is now appreciated that the frequency of adverse reactions is a sum of the function of the chemistry of drug molecules, a function of the biology of the cells, and a function of the genotype of patients. Therefore, *in vitro* model systems to look at the interplay between drug–protein conjugation factors, immunological mechanisms, and genetic factors are to be developed in order to define both chemical and biological variables that underpin serious idiosyncratic adverse reactions. It must be stressed that new *in vitro* model systems must be physiologically relevant to ensure that their outcome reflects what is happening in humans. Ultimately, the goal is to be able to investigate conjugate formation in order to predict adverse drug reactions. Considering the low incidence of these reactions, a prospective study would have to be conducted on a massive scale.

There is a growing list of drugs associated with idiosyncratic drug reactions. With the resurgence of drugs designed intentionally to covalently bind to their pharmacological targets, in order to increase potency, there is a need to be ever vigilant to the potential of drug to cause hypersensitivity during drug development. There are a set of clear but challenging objectives to be met for the progression of this field. However, it is certain, that this will be greatly assisted by the MS technology and techniques available today.

AUTHOR INFORMATION

Corresponding Author

*Tel: +44 151 7948386. E-mail: xlmeng@liv.ac.uk.

Author Contributions

[†]A.T. and J.C.W. contributed equally to this work.

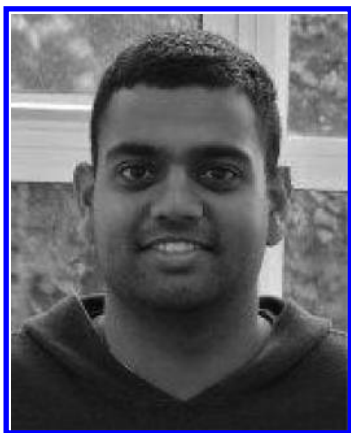
Funding

The work was supported by the Medical Research Council Centre for Drug Safety Science (Grant Number MR/L006758/1). A.T. is a Ph.D. student funded by GSK.

Notes

The authors declare no competing financial interest.

Biographies



Arun Tailor graduated with a degree in Pharmacology at the University of Liverpool in 2010. He has remained at this institution and since obtained a Masters of Research in Biomedical Sciences and Translational Medicine, and he is currently a Ph.D. Student at the MRC Centre for Drug Safety Science under a GSK funded studentship. His research involves investigating the chemical nature of drug–protein adducts which are presented by MHC molecules in drug hypersensitivity syndrome and drug induced liver injury. His project involves a multidisciplinary approach combining mass spectrometric techniques and T-cell culture assays.



James C. Waddington obtained a Master of Biological Sciences degree from the University of Liverpool in 2015, with honors in Microbiology. Previous research focuses included the implementation of novel techniques to analyze microbial metagenomics signatures in order to identify disease biomarkers, cellular responses to HIV viral infection, and toxin gene regulation in bacterial viruses. In 2015, James joined the MRC Centre for Drug Safety Science at the University of Liverpool as a Ph.D. student in pharmacology. Current research focusses surround the characterization of naturally processed MHC peptides that act as T cell antigens in patients with drug hypersensitivity.



Dr. Xiaoli Meng received her Ph.D. in medicinal chemistry from the University of Liverpool, with a focus on the synthesis of reactive drug metabolites. She is currently working in the MRC Centre of Drug Safety Science at the University of Liverpool as a postdoctoral fellow. Her research interests are drug metabolism, mass spectrometric characterization of covalent binding, and immune-mediated adverse drug reactions. She has published more than 20 research articles in various peer-reviewed journals.



Professor Kevin Park is Professor of Pharmacology, Head of the Institute of Translational Medicine at the University of Liverpool and Director of the UKRMP Safety Hub. He was the founding Director of the MRC Centre for Drug Safety Science. Professor Park is a Fellow of the Royal College of Physicians and a Fellow of the Academy of Medical Sciences. His work bridges “molecule-to-man” and back again for prediction of adverse drug reactions based on the chemistry of the drug and the identification of susceptible individuals. More recently, his work has expanded to understand the safety and efficacy of regenerative medicines.

ABBREVIATIONS

HSA, human serum albumin; MS, mass spectrometry “or” mass spectrometric; GSTP, glutathione S-transferase pi; AMS, accelerated mass spectrometry; LSC, liquid scintillation

counting; MALDI-TOF, matrix-assisted laser desorption/ionization time-of-flight; QTOF, quadrupole time-of-flight; MRM, multiple reaction monitoring; MW, molecular weight; TMT, tandem mass tagging; iTRAQ, isobaric tag for relative and absolute quantification; RMs, reactive metabolites; MBI, mechanism based inhibition; NVP, nevirapine; HIV, human immunodeficiency virus; APAP, acetaminophen; NAPQI, *N*-acetyl-*p*-benzoquinoneimine; β -LGA, beta lactoglobulin A; ALF, acute liver failure; ABC, abacavir; ABCA, abacavir α,β -unsaturated aldehydes; EGFR, epidermal growth factor receptor; HER, human epidermal growth factor receptor; SMX, sulfamethoxazole; SMX-NOH, sulfamethoxazole hydroxylamine; SMXNO, sulfamethoxazole nitroso; NSAIDs, non-steroidal anti-inflammatory drugs; AGs, β -1-*O*-acyl glucuronides; acyl-CoAs, thioester derivatives; NRF2, nuclear factor (erythroid-derived 2)-like 2; KEAP, Kelch-like ECH-associated protein; PD-1, programmed cell death protein 1; HLA, human leukocyte antigen; HLA DR, human leukocyte antigen - antigen D related; IgE, immunoglobulin E; DTR, delayed type reaction; DAMPs, damage associated molecular patterns; TLRs, toll-like receptors; NLRs, nucleotide-binding oligomerization domain like receptors, NAPL3, NACHT, LRR, and PYD domains-containing protein 3; DC, dendritic cell; DNCB, 2,4-dinitrochlorobenzene and; EBV, Epstein–Barr virus transformed B cell; ADRs, adverse drug reactions

REFERENCES

- (1) Potashman, M. H., and Duggan, M. E. (2009) Covalent modifiers: an orthogonal approach to drug design. *J. Med. Chem.* 52, 1231–1246.
- (2) Singh, J., Petter, R. C., Baillie, T. A., and Whitty, A. (2011) The resurgence of covalent drugs. *Nat. Rev. Drug Discovery* 10, 307–317.
- (3) Kalgutkar, A. S., and Dalvie, D. K. (2012) Drug discovery for a new generation of covalent drugs. *Expert Opin. Drug Discovery* 7, 561–581.
- (4) Miller, J. A., and Miller, E. C. (1947) The metabolism and carcinogenicity of *p*-dimethylaminoazobenzene and related compounds in the rat. *Cancer Res.* 7, 39–41.
- (5) Schneider, C. H., and De Weck, A. L. (1965) A new chemical aspect of penicillin allergy: the direct reaction of penicillin with ϵ -amino groups. *Nature* 208, 57–59.
- (6) Kitteringham, N. R., Maggs, J. L., Newby, S., and Park, B. K. (1985) Drug-protein conjugates-VIII. The metabolic fate of the dinitrophenyl hapten conjugated to albumin. *Biochem. Pharmacol.* 34, 1763–1771.
- (7) Schreiber, S. L. (2000) Target-Oriented and Diversity-Oriented Organic Synthesis in Drug Discovery. *Science* 287, 1964–1969.
- (8) Adkins, J. N. (2002) Toward a Human Blood Serum Proteome: Analysis By Multidimensional Separation Coupled With Mass Spectrometry. *Mol. Cell. Proteomics* 1, 947–955.
- (9) Steel, L. F., Trotter, M. G., Nakajima, P. B., Mattu, T. S., Gonye, G., and Block, T. (2003) Efficient and specific removal of albumin from human serum samples. *Mol. Cell. Proteomics* 2, 262–270.
- (10) Elsadek, B., and Kratz, F. (2012) Impact of albumin on drug delivery - New applications on the horizon. *J. Controlled Release* 157, 4–28.
- (11) Zheng, Y. R., Suntharalingam, K., Johnstone, T. C., Yoo, H., Lin, W., Brooks, J. G., and Lippard, S. J. (2014) Pt (IV) prodrugs designed to bind non-covalently to human serum albumin for drug delivery. *J. Am. Chem. Soc.* 136, 8790–8798.
- (12) Garzon, D., Ariza, A., Regazzoni, L., Clerici, R., Altomare, A., Sirtori, F. R., Carini, M., Torres, M. J., Perez-Sala, D., and Aldini, G. (2014) Mass spectrometric strategies for the identification and characterization of human serum albumin covalently adducted by amoxicillin: Ex Vivo Studies. *Chem. Res. Toxicol.* 27, 1566–1574.
- (13) Jenkins, R. E., Yaseen, F. S., Monshi, M. M., Whitaker, P., Meng, X., Farrell, J., Hamlett, J., Sanderson, J. P., El-Ghaiesh, S., Peckham, D., Pirmohamed, M., Park, B. K., and Naisbitt, D. J. (2013) β -lactam antibiotics form distinct haptenic structures on albumin and activate drug-specific T-lymphocyte responses in multiallergic patients with cystic fibrosis. *Chem. Res. Toxicol.* 26, 963–975.
- (14) Batchelor, F. R., Dewdney, J. M., and Gazzard, D. (1965) Penicillin Allergy: The Formation of the Penicilloyl Determinant. *Nature* 206, 362–364.
- (15) Levine, B. B., and Ovary, Z. (1961) Studies on the mechanism of the formation of the penicillin antigen: III. The N-(D-alpha-benzylpenicilloyl) group as an antigenic determinant responsible for hypersensitivity to penicillin G. *J. Exp. Med.* 114, 875–940.
- (16) Meng, X., Jenkins, R. E., Berry, N. G., Maggs, J. L., Farrell, J., Lane, C. S., Stachulski, A. V., French, N. S., Naisbitt, D. J., Pirmohamed, M., and Park, B. K. (2011) Direct evidence for the formation of diastereoisomeric benzylpenicilloyl haptens from benzylpenicillin and benzylpenicillenic acid in patients. *J. Pharmacol. Exp. Ther.* 338, 841–849.
- (17) Fang, X., and Zhang, W. W. (2008) Affinity separation and enrichment methods in proteomic analysis. *J. Proteomics* 71, 284–303.
- (18) Jenkins, R. E., Meng, X., Elliott, V. L., Kitteringham, N. R., Pirmohamed, M., and Park, B. K. (2009) Characterisation of flucloxacillin and 5-hydroxymethyl flucloxacillin haptenated HSA in vitro and in vivo. *Proteomics: Clin. Appl.* 3, 720–729.
- (19) El-Ghaiesh, S., Monshi, M. M., Whitaker, P., Jenkins, R., Meng, X., Farrell, J., Elsheikh, A., Peckham, D., French, N., Pirmohamed, M., Park, B. K., and Naisbitt, D. J. (2012) Characterization of the antigen specificity of T-cell clones from piperacillin-hypersensitive patients with cystic fibrosis. *J. Pharmacol. Exp. Ther.* 341, 597–610.
- (20) Isin, E. M., Elmore, C. S., Nilsson, G. N., Thompson, R. A., and Weidolf, L. (2012) Use of radiolabeled compounds in drug metabolism and pharmacokinetic studies. *Chem. Res. Toxicol.* 25, 532–542.
- (21) Grunwald, H., Hargreaves, P., Gebhardt, K., Klauer, D., Serafin, A., Schmitt-Hoffmann, A., Schleimer, M., Schlotterbeck, G., and Wind, M. (2013) Experiments for a systematic comparison between stable-isotope-(deuterium) labeling and radio-(^{14}C) labeling for the elucidation of the in vitro metabolic pattern of pharmaceutical drugs. *J. Pharm. Biomed. Anal.* 85, 138–144.
- (22) Marques Bernardoni, F., Pollack, S., and Helmy, R. R. (2014) Development of an automated system for preparation of liquid scintillation counting samples for radiolabeled pharmaceuticals. *J. Labelled Compd. Radiopharm.* 57, 121–124.
- (23) Bronić, I. K., Barešić, J., Horvatinčić, N., and Sironić, A. (2016) Determination of biogenic component in liquid fuels by the ^{14}C direct LSC method by using quenching properties of modern liquids for calibration. *Radiat. Phys. Chem.*, 1–6.
- (24) Chandrasekaran, A., Shen, L., Lockhead, S., Oganessian, A., Wang, J., and Scatina, J. (2010) Reversible covalent binding of neratinib to human serum albumin in vitro. *Drug Metab. Lett.* 4, 220–227.
- (25) Rodrigues, A. V. M., Rollison, H. E., Martin, S., Sarda, S., Schulz-Utermoehl, T., Stahl, S., Gustafsson, F., Eakins, J., Kenna, J. G., and Wilson, I. D. (2013) In vitro exploration of potential mechanisms of toxicity of the human hepatotoxic drug fenclozic acid. *Arch. Toxicol.* 87, 1569–1579.
- (26) Koen, Y. M., Sarma, D., Hajovsky, H., Galeva, N. A., Williams, T. D., Staudinger, J. L., and Hanzlik, R. P. (2013) Protein targets of thioacetamide metabolites in rat hepatocytes. *Chem. Res. Toxicol.* 26, 564–574.
- (27) McVey, C. E., Walsh, M. A., Dodson, G. G., Wilson, K. S., and Brannigan, J. A. (2001) Crystal structures of penicillin acylase enzyme-substrate complexes: structural insights into the catalytic mechanism. *J. Mol. Biol.* 313, 139–150.
- (28) Domon, B., and Aebersold, R. (2006) Mass spectrometry and protein analysis. *Science* 312, 212–217.
- (29) Angel, T. E., Aryal, U. K., Hengel, S. M., Baker, E. S., Kelly, R. T., Robinson, E. W., and Smith, R. D. (2012) Mass spectrometry-based proteomics: existing capabilities and future directions. *Chem. Soc. Rev.* 41, 3912–3928.
- (30) Bogdanov, B., and Smith, R. D. (2005) Proteomics by FTICR mass spectrometry: Top down and bottom up. *Mass Spectrom. Rev.* 24, 168–200.

- (31) Zhang, H., and Ge, Y. (2011) Comprehensive analysis of protein modifications by top-down mass spectrometry. *Circ.: Cardiovasc. Genet.* 4, 711.
- (32) Siuti, N., and Kelleher, N. L. (2007) Decoding protein modifications using top-down mass spectrometry. *Nat. Methods* 4, 817–821.
- (33) Yamada, M., Murakami, K., Wallingford, J. C., and Yuki, Y. (2002) Identification of low-abundance proteins of bovine colostrum and mature milk using two-dimensional electrophoresis followed by microsequencing and mass spectrometry. *Electrophoresis* 23, 1153–1160.
- (34) Calderaro, A., Arcangeletti, M., Rodighiero, I., Buttrini, M., Gorrini, C., Motta, F., Germini, D., Medici, M., Chezzi, C., and De Conto, F. (2014) Matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry applied to virus identification. *Sci. Rep.* 4, 6803.
- (35) De Carolis, E., Vella, A., Vaccaro, L., Torelli, R., Spanu, T., Fiori, B., Posteraro, B., and Sanguinetti, M. (2014) Application of MALDI-TOF mass spectrometry in clinical diagnostic microbiology. *J. Infect. Dev. Countries* 8, 1081–1088.
- (36) Zubarev, R. A., Horn, D. M., Fridriksson, E. K., Kelleher, N. L., Kruger, N. A., Lewis, M. A., Carpenter, B. K., and McLafferty, F. W. (2000) Electron capture dissociation for structural characterization of multiply charged protein cations. *Anal. Chem.* 72, 563–573.
- (37) Kelleher, N. L., Lin, H. Y., Valaskovic, G. A., Aaserud, D. J., Fridriksson, E. K., and McLafferty, F. W. (1999) Top Down versus Bottom Up Protein Characterization by Tandem High-Resolution Mass Spectrometry. *J. Am. Chem. Soc.* 121, 806–812.
- (38) Helm, D., Vissers, J. P. C., Hughes, C. J., Hahne, H., Ruprecht, B., Pahl, F., Grzyb, A., Richardson, K., Wildgoose, J., Maier, S. K., Marx, H., Wilhelm, M., Becher, I., Lemeer, S., Bantscheff, M., Langridge, J. I., and Kuster, B. (2014) Ion mobility tandem mass spectrometry enhances performance of bottom-up proteomics. *Mol. Cell. Proteomics* 13, 3709–3715.
- (39) Liang, S., Li, X., Cao, M., Xie, J., Chen, P., and Huang, R. (2000) Identification of venom proteins of spider *S. huwena* on Two-dimensional electrophoresis gel by N-terminal microsequencing and mass spectrometric peptide mapping. *J. Protein Chem.* 19, 225–229.
- (40) Bagshaw, R. D., Callahan, J. W., and Mahuran, D. J. (2000) Desalting of in-gel-digested protein sample with mini-C18 columns for matrix-assisted laser desorption ionization time of flight peptide mass fingerprinting. *Anal. Biochem.* 284, 432–435.
- (41) de Hoffmann, E. (1996) Tandem mass spectrometry: A primer. *J. Mass Spectrom.* 31, 129–137.
- (42) Gillette, M. A., and Carr, S. A. (2013) Quantitative analysis of peptides and proteins in biomedicine by targeted mass spectrometry. *Nat. Methods* 10, 28–34.
- (43) Mauri-Hellweg, D., Zanni, M., Frei, E., Bettens, F., Brander, C., Mauri, D., Padovan, E., Weltzien, H. U., and Pichler, W. J. (1996) Cross-reactivity of T cell lines and clones to beta-lactam antibiotics. *J. Immunol.* 157, 1071–1079.
- (44) Olsen, J. V., Ong, S. E., and Mann, M. (2004) Trypsin cleaves exclusively C-terminal to arginine and lysine residues. *Mol. Cell. Proteomics* 3, 608–614.
- (45) Thiede, B., Lamer, S., Mattow, J., Siejak, F., Dimmler, C., Rudel, T., and Jungblut, P. R. (2000) Analysis of missed cleavage sites, tryptophan oxidation and N-terminal pyroglutamylation after in-gel tryptic digestion. *Rapid Commun. Mass Spectrom.* 14, 496–502.
- (46) Wiese, S., Reidegeld, K. A., Meyer, H. E., and Warscheid, B. (2007) Protein labeling by iTRAQ: A new tool for quantitative mass spectrometry in proteome research. *Proteomics* 7, 340–350.
- (47) Link, A. J., Eng, J., Schieltz, D. M., Carmack, E., Mize, G. J., Morris, D. R., Garvik, B. M., and Yates, J. R. (1999) Direct analysis of protein complexes using mass spectrometry. *Nat. Biotechnol.* 17, 676–682.
- (48) Pichler, P., Köcher, T., Holzmann, J., Mazanek, M., Taus, T., Ammerer, G., and Mechtler, K. (2010) Peptide labeling with isobaric tags yields higher identification rates using iTRAQ 4-plex compared to TMT 6-plex and iTRAQ 8-plex on LTQ orbitrap. *Anal. Chem.* 82, 6549–6558.
- (49) Wu, J., Warren, P., Shakey, Q., Sousa, E., Hill, A., Ryan, T. E., and He, T. (2010) Integrating titania enrichment, iTRAQ labeling, and orbitrap CID-HCD for global identification and quantitative analysis of phosphopeptides. *Proteomics* 10, 2224–2234.
- (50) Boehm, A. M., Pütz, S., Altenhöfer, D., Sickmann, A., and Falk, M. (2007) Precise protein quantification based on peptide quantification using iTRAQ. *BMC Bioinf.* 8, 214.
- (51) Gan, C. S., Chong, P. K., Pham, T. K., and Wright, P. C. (2007) Technical, experimental, and biological variations in isobaric tags for relative and absolute quantitation (iTRAQ). *J. Proteomic Res.* 6, 821–827.
- (52) Dayon, L., Hainard, A., Licker, V., Turck, N., Hochstrasser, D. F., Burkhard, P. R., Sanchez, J., and Kuhn, K. (2008) Relative quantification of proteins in human cerebrospinal fluids by MS/MS using 6-plex isobaric tags. *Anal. Chem.* 80, 2921–2931.
- (53) Zieske, L. R. (2006) A perspective on the use of iTRAQ reagent technology for protein complex and profiling studies. *J. Exp. Bot.* 57, 1501–1508.
- (54) Aggarwal, K., Choe, L. H., and Lee, K. H. (2006) Shotgun proteomics using the iTRAQ isobaric tags. *Briefings Funct. Genomics Proteomics* 5, 112–120.
- (55) Evans, D. C., Watt, A. P., Nicoll-Griffith, D. A., and Baillie, T. A. (2004) Drug-protein adducts: an industry perspective on minimizing the potential for drug bioactivation in drug discovery and development. *Chem. Res. Toxicol.* 17, 3–16.
- (56) Day, S. H., Mao, A., White, R., Schulz-Utermoehl, T., Miller, R., and Beconi, M. G. (2005) A semi-automated method for measuring the potential for protein covalent binding in drug discovery. *J. Pharmacol. Toxicol. Methods* 52, 278–285.
- (57) Park, B. K., Boobis, A., Clarke, S., Goldring, C. E. P., Jones, D., Kenna, J. G., Lambert, C., Lavery, H. G., Naisbitt, D. J., Nelson, S., Nicoll-Griffith, D. A., Obach, R. S., Routledge, P., Smith, D. A., Tweedie, D. J., Vermeulen, N., Williams, D. P., Wilson, I. D., and Baillie, T. A. (2011) Managing the challenge of chemically reactive metabolites in drug development. *Nat. Rev. Drug Discovery* 10, 292–306.
- (58) Baillie, T. A. (2008) Metabolism and Toxicity of Drugs. Two Decades of Progress in Industrial Drug Metabolism. *Chem. Res. Toxicol.* 21, 129–137.
- (59) Doss, G. A., and Baillie, T. A. (2006) Addressing metabolic activation as an integral component of drug design. *Drug Metab. Rev.* 38, 641–649.
- (60) Myers, S. R., Spinnato, J. A., and Pinorini, M. T. (1996) Chromatographic characterization of hemoglobin benzo[a]pyrene-7,8-diol-9,10-epoxide adducts. *Toxicol. Sci.* 29, 94–101.
- (61) Whitaker, P., Meng, X., Lavergne, S. N., El-Ghaiesh, S., Monshi, M., Earnshaw, C., Peckham, D., Gooi, J., Conway, S., Pirmohamed, M., Jenkins, R. E., Naisbitt, D. J., and Park, B. K. (2011) Mass spectrometric characterization of circulating and functional antigens derived from piperacillin in patients with cystic fibrosis. *J. Immunol.* 187, 200–211.
- (62) Ariza, A., Garzon, D., Abánades, D. R., de los Ríos, V., Vistoli, G., Torres, M. J., Carini, M., Aldini, G., and Pérez-Sala, D. (2012) Protein haptentation by amoxicillin: high resolution mass spectrometry analysis and identification of target proteins in serum. *J. Proteomics* 77, 504–520.
- (63) Kang, P., Liao, M., Wester, M. R., Leeder, S., Pearce, R. E., and Correia, M. A. (2008) CYP3A4-Mediated carbamazepine (CBZ) metabolism: formation of a covalent CBZ-CYP3A4 adduct and alteration of the enzyme kinetic profile. *Drug Metab. Dispos.* 36, 490–499.
- (64) Lu, W., and Uetrecht, J. P. (2008) Peroxidase-mediated bioactivation of hydroxylated metabolites of carbamazepine and phenytoin. *Drug Metab. Dispos.* 36, 1624–1636.
- (65) Yip, V., Maggs, J., Meng, X., Marson, A., Park, B. K., and Pirmohamed, M. (2014) Covalent adduction of carbamazepine 10, 11-epoxide with human serum albumin and glutathione S-transferase pi: implications for carbamazepine hypersensitivity. *Lancet* 383, S114.
- (66) Chen, J., Mannargudi, B. M., Xu, L., and Uetrecht, J. (2008) Demonstration of the metabolic pathway responsible for nevirapine-induced skin rash. *Chem. Res. Toxicol.* 21, 1862–1870.

- (67) Wen, B., Chen, Y., and Fitch, W. L. (2009) Metabolic activation of nevirapine in human liver microsomes: dehydrogenation and inactivation of cytochrome P450 3A4. *Drug Metab. Dispos.* 37, 1557–1562.
- (68) Srivastava, A., Lian, L.-Y., Maggs, J. L., Chaponda, M., Pirmohamed, M., Williams, D. P., and Park, B. K. (2010) Quantifying the metabolic activation of nevirapine in patients by integrated applications of NMR and mass spectrometries. *Drug Metab. Dispos.* 38, 122–132.
- (69) Caixas, U., Antunes, A. M., Marinho, A. T., Godinho, A. L., Grilo, N. M., Marques, M. M., Oliveira, M. C., Branco, T., Monteiro, E. C., and Pereira, S. A. (2012) Evidence for nevirapine bioactivation in man: searching for the first step in the mechanism of nevirapine toxicity. *Toxicology* 301, 33–39.
- (70) Sharma, A. M., Klarskov, K., and Uetrecht, J. (2013) Nevirapine bioactivation and covalent binding in the skin. *Chem. Res. Toxicol.* 26, 410–421.
- (71) Sharma, A. M., Novalen, M., Tanino, T., and Uetrecht, J. P. (2013) 12-OH-nevirapine sulfate, formed in the skin, is responsible for nevirapine-induced skin rash. *Chem. Res. Toxicol.* 26, 817–827.
- (72) Meng, X., Howarth, A., Earnshaw, C. J., Jenkins, R. E., French, N. S., Back, D. J., Naisbitt, D. J., and Park, B. K. (2013) Detection of drug bioactivation in vivo: mechanism of nevirapine-albumin conjugate formation in patients. *Chem. Res. Toxicol.* 26, 575–583.
- (73) Heard, K., Green, J. L., Anderson, V., Bucher-Bartelson, B., and Dart, R. C. (2016) Paracetamol (acetaminophen) protein adduct concentrations during therapeutic dosing. *Br. J. Clin. Pharmacol.* 81, 562–568.
- (74) Switzar, L., Kwast, L. M., Lingeman, H., Giera, M., Pieters, R. H. H., and Niessen, W. M. A. (2013) Identification and quantification of drug-albumin adducts in serum samples from a drug exposure study in mice. *J. Chromatogr. B: Anal. Technol. Biomed. Life Sci.* 917–918, 53–61.
- (75) Cook, S. F., King, A. D., Chang, Y., Murray, G. J., Norris, H. K., Dart, R. C., Green, J. L., Curry, S. C., Rollins, D. E., and Wilkins, D. G. (2015) Quantification of a biomarker of acetaminophen protein adducts in human serum by high-performance liquid chromatography-electrospray ionization-tandem mass spectrometry: clinical and animal model applications. *J. Chromatogr. B: Anal. Technol. Biomed. Life Sci.* 985, 131–141.
- (76) Davern, T. J., James, L. P., Hinson, J. A., Polson, J., Larson, A. M., Fontana, R. J., Lalani, E., Munoz, S., Shakil, A. O., Lee, W. M., Acute Liver Failure Study Group, Davern, T. J., 2nd, James, L. P., Hinson, J. A., Polson, J., Larson, A. M., Fontana, R. J., Lalani, E., Munoz, S., Shakil, A. O., Lee, W. M., and Acute Liver Failure Study, G. (2006) Measurement of serum acetaminophen-protein adducts in patients with acute liver failure. *Gastroenterology* 130, 687–694.
- (77) Golizeh, M., LeBlanc, A., and Sleno, L. (2015) Identification of Acetaminophen Adducts of Rat Liver Microsomal Proteins using 2D-LC-MS/MS. *Chem. Res. Toxicol.* 28, 2142–2150.
- (78) Lohmann, W., Hayen, H., and Karst, U. (2008) Covalent protein modification by reactive drug metabolites using online electrochemistry/liquid chromatography/mass spectrometry. *Anal. Chem.* 80, 9714–9719.
- (79) James, L. P., Alonso, E. M., Hynan, L. S., Hinson, J. A., Davern, T. J., Lee, W. M., Squires, R. H., and Pediatric Acute Liver Failure Study Group (2006) Detection of acetaminophen protein adducts in children with acute liver failure of indeterminate cause. *Pediatrics* 118, e676–681.
- (80) Walsh, J. S., Reese, M. J., and Thurmond, L. M. (2002) The metabolic activation of abacavir by human liver cytosol and expressed human alcohol dehydrogenase isozymes. *Chem.-Biol. Interact.* 142, 135–154.
- (81) Charneira, C., Godinho, A. L. A., Oliveira, M. C., Pereira, S. A., Monteiro, E. C., Marques, M. M., and Antunes, A. M. M. (2011) Reactive aldehyde metabolites from the anti-HIV drug abacavir: amino acid adducts as possible factors in abacavir toxicity. *Chem. Res. Toxicol.* 24, 2129–2141.
- (82) Charneira, C., Grilo, N. M., Pereira, S. A., Godinho, A. L. A., Monteiro, E. C., Marques, M. M., and Antunes, A. M. M. (2012) N-terminal valine adduct from the anti-HIV drug abacavir in rat haemoglobin as evidence for abacavir metabolism to a reactive aldehyde in vivo. *Br. J. Pharmacol.* 167, 1353–1361.
- (83) Grilo, N. M., Antunes, A. M., Caixas, U., Marinho, A. T., Charneira, C., Conceição Oliveira, M., Monteiro, E. C., Matilde Marques, M., and Pereira, S. A. (2013) Monitoring abacavir bioactivation in humans: screening for an aldehyde metabolite. *Toxicol. Lett.* 219, 59–64.
- (84) Meng, X., Lawrenson, A. S., Berry, N. G., Maggs, J. L., French, N. S., Back, D. J., Khoo, S. H., Naisbitt, D. J., and Park, B. K. (2014) Abacavir forms novel cross-linking abacavir protein adducts in patients. *Chem. Res. Toxicol.* 27, 524–535.
- (85) Solca, F., Dahl, G., Zoephel, A., Bader, G., Sanderson, M., Klein, C., Kraemer, O., Himmelsbach, F., Haaksm, E., and Adolf, G. R. G. R. (2012) Target binding properties and cellular activity of afatinib (BIBW 2992), an irreversible ErbB family blocker. *J. Pharmacol. Exp. Ther.* 343, 342–350.
- (86) Wang, J., Li-Chan, X. X., Atherton, J., Deng, L., Espina, R., Yu, L., Horwatt, P., Ross, S., Lockhead, S., Ahmad, S., Chandrasekaran, A., Oganessian, A., Scatina, J., Mutlib, A., and Talaat, R. (2010) Characterization of HKI-272 covalent binding to human serum albumin. *Drug Metab. Dispos.* 38, 1083–1093.
- (87) Klaeger, S., Gohlke, B., Perrin, J., Gupta, V., Heinzlmeir, S., Helm, D., Qiao, H., Bergamini, G., Handa, H., Savitski, M. M., Bantscheff, M., Médard, G., Preissner, R., and Kuster, B. (2016) Chemical Proteomics Reveals Ferrochelatase as a Common Off-target of Kinase Inhibitors. *ACS Chem. Biol.* 11, 1245–1254.
- (88) Brown, A. P., Dunstan, R. W., Courtney, C. L., Criswell, K. A., and Graziano, M. J. (2008) Cutaneous lesions in the rat following administration of an irreversible inhibitor of erbB receptors, including the epidermal growth factor receptor. *Toxicol. Pathol.* 36, 410–419.
- (89) Roujeau, J. C., Mockenhaupt, M., Tahan, S. R., Henshaw, J., Martin, E. C., Harding, M., van Baelen, B., Bengtsson, L., Singhal, P., Kauffman, R. S., and Stern, R. S. (2013) Telaprevir-related dermatitis. *JAMA dermatology* 149, 152–158.
- (90) Kalgutkar, A. S., Dalvie, D. K., O'Donnell, J. P., Taylor, T. J., and Sahakian, D. C. (2002) On the diversity of oxidative bioactivation reactions on nitrogen-containing xenobiotics. *Curr. Drug Metab.* 3, 379–424.
- (91) Wang, Y., Peng, L., Bellamri, M., Langouët, S., and Turesky, R. J. (2015) Mass Spectrometric Characterization of Human Serum Albumin Adducts Formed with N-Oxidized Metabolites of 2-Amino-1-methylphenylimidazo[4,5-b]pyridine in Human Plasma and Hepatocytes. *Chem. Res. Toxicol.* 28, 1045–1059.
- (92) Schnyder, B., Burkhart, C., Schnyder-Frutig, K., von Greyerz, S., Naisbitt, D. J., Pirmohamed, M., Park, B. K., and Pichler, W. J. (2000) Recognition of sulfamethoxazole and its reactive metabolites by drug-specific CD4+ T cells from allergic individuals. *J. Immunol.* 164, 6647–6654.
- (93) Burkhart, C., von Greyerz, S., Depta, J. P., Naisbitt, D. J., Britschgi, M., Park, K. B., and Pichler, W. J. (2001) Influence of reduced glutathione on the proliferative response of sulfamethoxazole-specific and sulfamethoxazole-metabolite-specific human CD4+ T-cells. *Br. J. Pharmacol.* 132, 623–630.
- (94) Elsheikh, A., Castrejon, L., Laverne, S. N., Whitaker, P., Monshi, M., Callan, H., El-Ghaiesh, S., Farrell, J., Pichler, W. J., Peckham, D., Park, B. K., and Naisbitt, D. J. (2011) Enhanced antigenicity leads to altered immunogenicity in sulfamethoxazole-hypersensitive patients with cystic fibrosis. *J. Allergy Clin. Immunol.* 127, 1543–1551.e3.
- (95) Manchanda, T., Hess, D., Dale, L., Ferguson, S. G., and Rieder, M. J. (2002) Haptenation of sulfonamide reactive metabolites to cellular proteins. *Mol. Pharmacol.* 62, 1011–1026.
- (96) Naisbitt, D. J., Farrell, J., Gordon, S. F., Maggs, J. L., Burkhart, C., Pichler, W. J., Pirmohamed, M., and Park, B. K. (2002) Covalent binding of the nitroso metabolite of sulfamethoxazole leads to toxicity and major histocompatibility complex-restricted antigen presentation. *Mol. Pharmacol.* 62, 628–637.
- (97) Callan, H. E., Jenkins, R. E., Maggs, J. L., Laverne, S. N., Clarke, S. E., Naisbitt, D. J., and Park, B. K. (2009) Multiple adduction reactions of nitroso sulfamethoxazole with cysteinyl residues of peptides and

- proteins: implications for hapten formation. *Chem. Res. Toxicol.* 22, 937–948.
- (98) Roychowdhury, S., Vyas, P. M., and Svensson, C. K. (2007) Formation and uptake of arylhydroxylamine-haptenated proteins in human dendritic cells. *Drug Metab. Dispos.* 35, 676–681.
- (99) Roychowdhury, S., Cram, A. E., Aly, A., and Svensson, C. K. (2007) Detection of haptenated proteins in organotypic human skin explant cultures exposed to dapsone. *Drug Metab. Dispos.* 35, 1463–1465.
- (100) Stachulski, A. V., Harding, J. R., Lindon, J. C., Maggs, J. L., Park, B. K., and Wilson, I. D. (2006) Acyl glucuronides: biological activity, chemical reactivity, and chemical synthesis. *J. Med. Chem.* 49, 6931–6945.
- (101) Stepan, A. F., Walker, D. P., Bauman, J., Price, D. A., Baillie, T. A., Kalgutkar, A. S., and Aleo, M. D. (2011) Structural alert/reactive metabolite concept as applied in medicinal chemistry to mitigate the risk of idiosyncratic drug toxicity: a perspective based on the critical examination of trends in the top 200 drugs marketed in the United States. *Chem. Res. Toxicol.* 24, 1345–1410.
- (102) Ding, A., Zia-Amirhosseini, P., McDonagh, A. F., Burlingame, A. L., and Benet, L. Z. (1995) Reactivity of tolmetin glucuronide with human serum albumin. Identification of binding sites and mechanisms of reaction by tandem mass spectrometry. *Drug Metab. Dispos.* 23, 369–376.
- (103) Qiu, Y., Burlingame, A. L., and Benet, L. Z. (1998) Mechanisms for covalent binding of benoxaprofen glucuronide to human serum albumin. Studies By tandem mass spectrometry. *Drug Metab. Dispos.* 26, 246–256.
- (104) Kenny, J. R., Maggs, J. L., Meng, X., Sinnott, D., Clarke, S. E., Park, B. K., and Stachulski, A. V. (2004) Syntheses and characterization of the acyl glucuronide and hydroxy metabolites of diclofenac. *J. Med. Chem.* 47, 2816–2825.
- (105) Hammond, T. G., Meng, X., Jenkins, R. E., Maggs, J. L., Castelazo, A. S., Regan, S. L., Bennett, S. N. L., Earnshaw, C. J., Aithal, G. P., Pande, I., Kenna, J. G., Stachulski, A. V., Park, B. K., and Williams, D. P. (2014) Mass spectrometric characterization of circulating covalent protein adducts derived from a drug acyl glucuronide metabolite: multiple albumin adductions in diclofenac patients. *J. Pharmacol. Exp. Ther.* 350, 387–402.
- (106) Dong, J. Q., Liu, J., and Smith, P. C. (2005) Role of benoxaprofen and flunoxaprofen acyl glucuronides in covalent binding to rat plasma and liver proteins in vivo. *Biochem. Pharmacol.* 70, 937–948.
- (107) Skonberg, C., Olsen, J., Madsen, K. G., Hansen, S. H., and Grillo, M. P. (2008) Metabolic activation of carboxylic acids. *Expert Opin. Drug Metab. Toxicol.* 4, 425–438.
- (108) Darnell, M., Breitholtz, K., Isin, E. M., Jurva, U., and Weidolf, L. (2015) Significantly Different Covalent Binding of Oxidative Metabolites, Acyl Glucuronides, and S-Acyl CoA Conjugates Formed from Xenobiotic Carboxylic Acids in Human Liver Microsomes. *Chem. Res. Toxicol.* 28, 886–896.
- (109) Olsen, J., Bjørnsdóttir, I., and Honoré Hansen, S. (2003) Identification of coenzyme A-related tolmetin metabolites in rats: relationship with reactive drug metabolites. *Xenobiotica* 33, 561–570.
- (110) Olsen, J., Li, C., Bjørnsdóttir, I., Sidenius, U., Hansen, S. H., and Benet, L. Z. (2005) In vitro and in vivo studies on acyl-coenzyme A-dependent bioactivation of zomepirac in rats. *Chem. Res. Toxicol.* 18, 1729–1736.
- (111) Olsen, J., Bjørnsdóttir, I., Tjørnelund, J., and Honoré Hansen, S. (2002) Chemical reactivity of the naproxen acyl glucuronide and the naproxen coenzyme A thioester towards bionucleophiles. *J. Pharm. Biomed. Anal.* 29, 7–15.
- (112) Grillo, M. P., Tadano Lohr, M., and Wait, J. C. M. (2012) Metabolic activation of mefenamic acid leading to mefenamyl-S-acyl-glutathione adduct formation in vitro and in vivo in rat. *Drug Metab. Dispos.* 40, 1515–1526.
- (113) Knights, K. M., Sykes, M. J., and Miners, J. O. (2007) Amino acid conjugation: contribution to the metabolism and toxicity of xenobiotic carboxylic acids. *Expert Opin. Drug Metab. Toxicol.* 3, 159–168.
- (114) Castillo, M., Lam, Y. W., Dooley, M. A., Stahl, E., and Smith, P. C. (1995) Disposition and covalent binding of ibuprofen and its acyl glucuronide in the elderly. *Clin. Pharmacol. Ther.* 57, 636–644.
- (115) Regan, S. L., Maggs, J. L., Hammond, T. G., Lambert, C., Williams, D. P., and Park, B. K. (2010) Acyl glucuronides: the good, the bad and the ugly. *Biopharm. Drug Dispos.* 31, 367–395.
- (116) Uetrecht, J., and Naisbitt, D. J. (2013) Idiosyncratic adverse drug reactions: current concepts. *Pharmacol. Rev.* 65, 779–808.
- (117) Beraldo, D. O., Melo, J. F., Bonfim, A. V., Teixeira, A. A., Teixeira, R. A., and Duarte, A. L. (2013) Acute cholestatic hepatitis caused by amoxicillin/clavulanate. *World J. Gastroenterol.* 19, 8789–8792.
- (118) Warrington, R. J., Tse, K. S., Gorski, B. A., Schwenk, R., and Sehon, A. H. (1978) Evaluation of isoniazid-associated hepatitis by immunological tests. *Clin. Exp. Immunol.* 32, 97–104.
- (119) Fontana, R. J. (2014) Pathogenesis of idiosyncratic drug-induced liver injury and clinical perspectives. *Gastroenterology* 146, 914–928.
- (120) Macherey, A., and Dansette, P. M. (2008) Biotransformations Leading to Toxic Metabolites, in *The Practice of Medicinal Chemistry* (Wermuth, C. G., Ed.) 3rd ed., pp 674–696, Elsevier, Amsterdam, The Netherlands.
- (121) Nguyen, T., Yang, C. S., and Pickett, C. B. (2004) The pathways and molecular mechanisms regulating Nrf2 activation in response to chemical stress. *Free Radical Biol. Med.* 37, 433–441.
- (122) Carey, M. A., and van Pelt, F. N. (2005) Immunochemical detection of flucloxacillin adduct formation in livers of treated rats. *Toxicology* 216, 41–48.
- (123) Davidson, D. G., and Eastham, W. N. (1966) Acute liver necrosis following overdose of paracetamol. *Br. Med. J.* 2, 497–499.
- (124) Dahlin, D. C., Miwa, G. T., Lu, A. Y., and Nelson, S. D. (1984) N-acetyl-p-benzoquinone imine: a cytochrome P-450-mediated oxidation product of acetaminophen. *Proc. Natl. Acad. Sci. U. S. A.* 81, 1327–1331.
- (125) Mitchell, J. R., Jollow, D. J., Potter, W. Z., Gillette, J. R., and Brodie, B. B. (1973) Acetaminophen-induced hepatic necrosis. IV. Protective role of glutathione. *J. Pharmacol. Exp. Ther.* 187, 211–217.
- (126) Corcoran, G. B., Todd, E. L., Racz, W. J., Hughes, H., Smith, C. V., and Mitchell, J. R. (1985) Effects of N-acetylcysteine on the disposition and metabolism of acetaminophen in mice. *J. Pharmacol. Exp. Ther.* 232, 857–863.
- (127) Roberts, D. W., Bucci, T. J., Benson, R. W., Warbritton, A. R., McRae, T. A., Pumford, N. R., and Hinson, J. A. (1991) Immunohistochemical localization and quantification of the 3-(cystein-S-yl)-acetaminophen protein adduct in acetaminophen hepatotoxicity. *Am. J. Pathol.* 138, 359–371.
- (128) Stamper, B. D. (2015) Transcriptional profiling of reactive metabolites for elucidating toxicological mechanisms: a case study of quinoneimine-forming agents. *Drug Metab. Rev.* 47, 45–55.
- (129) Heidari, R., Babaei, H., and Eghbal, M. (2014) Amodiaquine-induced toxicity in isolated rat hepatocytes and the cytoprotective effects of taurine and/or N-acetyl cysteine. *Res. Pharm. Sci.* 9, 97–105.
- (130) Farombi, E. O., Olowu, B. I., and Emerole, G. O. (2000) Effect of three structurally related antimalarial drugs on liver microsomal components and lipid peroxidation in rats. *Comp. Biochem. Physiol., Part C: Pharmacol., Toxicol. Endocrinol.* 126, 217–224.
- (131) Naisbitt, D. J., Williams, D. P., O'Neill, P. M., Maggs, J. L., Willock, D. J., Pirmohamed, M., and Park, B. K. (1998) Metabolism-dependent neutrophil cytotoxicity of amodiaquine: A comparison with pyronaridine and related antimalarial drugs. *Chem. Res. Toxicol.* 11, 1586–1595.
- (132) Metushi, I. G., Hayes, M. A., and Uetrecht, J. (2015) Treatment of PD-1(–/–) mice with amodiaquine and anti-CTLA4 leads to liver injury similar to idiosyncratic liver injury in patients. *Hepatology* 61, 1332–1342.
- (133) Wang, X., Sun, R., Chen, Y., Lian, Z., Wei, H., and Tian, Z. (2015) Regulatory T cells ameliorate acetaminophen-induced immune-mediated liver injury. *Int. Immunopharmacol.* 25, 293–301.
- (134) Tailor, A., Faulkner, L., Naisbitt, D., and Park, B. (2015) The chemical, genetic and immunological basis of idiosyncratic drug-induced liver injury. *Hum. Exp. Toxicol.* 34, 1310–1317.

- (135) Mansuy, D. (1997) Molecular structure and hepatotoxicity: compared data about two closely related thiophene compounds. *J. Hepatol.* 26, 22–25.
- (136) Neuberger, J., and Williams, R. (1989) Immune mechanisms in tienilic acid associated hepatotoxicity. *Gut* 30, 515–519.
- (137) Robin, M. A., Maratrat, M., Le Roy, M., Le Breton, F. P., Bonierbale, E., Dansette, P., Ballet, F., Mansuy, D., and Pessayre, D. (1996) Antigenic targets in tienilic acid hepatitis. Both cytochrome P450 2C11 and 2C11-tienilic acid adducts are transported to the plasma membrane of rat hepatocytes and recognized by human sera. *J. Clin. Invest.* 98, 1471–1480.
- (138) Takakusa, H., Wahlin, M. D., Zhao, C., Hanson, K. L., New, L. S., Chan, E. C. Y., and Nelson, S. D. (2011) Metabolic intermediate complex formation of human cytochrome P450 3A4 by lapatinib. *Drug Metab. Dispos.* 39, 1022–1030.
- (139) Teng, W. C., Oh, J. W., New, L. S., Wahlin, M. D., Nelson, S. D., Ho, H. K., and Chan, E. C. Y. (2010) Mechanism-based inactivation of cytochrome P450 3A4 by lapatinib. *Mol. Pharmacol.* 78, 693–703.
- (140) Parham, L. R., Briley, L. P., Li, L., Shen, J., Newcombe, P. J., King, K. S., Slater, A. J., Dilthey, A., Iqbal, Z., McVean, G., Cox, C. J., Nelson, M. R., and Spraggs, C. F. (2016) Comprehensive genome-wide evaluation of lapatinib-induced liver injury yields a single genetic signal centered on known risk allele HLA-DRB1*07:01. *Pharmacogenomics J.* 16, 180–185.
- (141) Gomes, E. R., and Demoly, P. (2005) Epidemiology of hypersensitivity drug reactions. *Curr. Opin. Allergy Clin. Immunol.* 5, 309–316.
- (142) Bell, L. N., and Chalasani, N. (2009) Epidemiology of idiosyncratic drug-induced liver injury. *Semin. Liver Dis.* 29, 337–347.
- (143) Ariza, A., Barrionuevo, E., Mayorga, C., Montañez, M. I., Perez-Inestrosa, E., Ruiz-Sánchez, A., Rodríguez-Guéant, R. M., Fernández, T. D., Guéant, J. L., Torres, M. J., and Blanca, M. (2014) IgE to penicillins with different specificities can be identified by a multipeptide macromolecule: Bihaptenic penicillin structures and IgE specificities. *J. Immunol. Methods* 406, 43–50.
- (144) Fernandez, T. D., Mayorga, C., Ariza, A., Corzo, J. L., and Torres, M. J. (2014) Allergic reactions to antibiotics in children. *Curr. Opin. Allergy Clin. Immunol.* 14, 278–285.
- (145) Daftarian, M. P., Filion, L. G., Cameron, W., Conway, B., Roy, R., Tropper, F., and Diaz-Mitoma, F. (1995) Immune response to sulfamethoxazole in patients with AIDS. *Clin. Diagn. Lab. Immunol.* 2, 199–204.
- (146) Blanca, M., Perez, E., Garcia, J., Miranda, A., Fernandez, J., Vega, J. M., Terrados, S., Avila, M., Martin, A., and Suau, R. (1988) Anaphylaxis to amoxycillin but good tolerance for benzyl penicillin. In vivo and in vitro studies of specific IgE antibodies. *Allergy* 43, 508–510.
- (147) Landsteiner, K., and Jacobs, J. (1935) Studies on the sensitization of animals with simple chemical compounds. *J. Exp. Med.* 61, 643–656.
- (148) Martin, S. F., Esser, P. R., Weber, F. C., Jakob, T., Freudenberg, M. A., Schmidt, M., and Goebeler, M. (2011) Mechanisms of chemical-induced innate immunity in allergic contact dermatitis. *Allergy* 66, 1152–1163.
- (149) Illing, P. T., Vivian, J. P., Dudek, N. L., Kostenko, L., Chen, Z., Bharadwaj, M., Miles, J. J., Kjer-Nielsen, L., Gras, S., Williamson, N. A., Burrows, S. R., Purcell, A. W., Rossjohn, J., and McCluskey, J. (2012) Immune self-reactivity triggered by drug-modified HLA-peptide repertoire. *Nature* 486, 554–558.
- (150) Maher, J. J. (2009) DAMPs ramp up drug toxicity. *J. Clin. Invest.* 119, 246–249.
- (151) Szabo, G., and Csak, T. (2012) Review Inflammasomes in liver diseases. *J. Hepatol.* 57, 642–654.
- (152) Imaeda, A. B., Watanabe, A., Sohail, M. A., Mahmood, S., Mohamadnejad, M., Sutterwala, F. S., Flavell, R. A., and Mehal, W. Z. (2009) Acetaminophen-induced hepatotoxicity in mice is dependent on Tlr9 and the Nalp3 inflammasome. *J. Clin. Invest.* 119, 305–314.
- (153) Schmidt, M., Raghavan, B., Müller, V., Vogl, T., et al. (2010) Crucial role for human Toll-like receptor 4 in the development of contact allergy to nickel. *Nat. Immunol.* 11, 814–819.
- (154) El Ali, Z., Gerbeix, C., Hemon, P., Esser, P. R., Martin, S. F., Pallardy, M., and Kerdine-Römer, S. (2013) Allergic skin inflammation induced by chemical sensitizers is controlled by the transcription factor Nrf2. *Toxicol. Sci.* 134, 39–48.
- (155) Copple, I. M., Goldring, C. E., Jenkins, R. E., Chia, A. J. L., Randle, L. E., Hayes, J. D., Kitteringham, N. R., and Park, B. K. (2008) The hepatotoxic metabolite of acetaminophen directly activates the keap1-Nrf2 cell defense system. *Hepatology* 48, 1292–1301.
- (156) Weltzien, H. U., Moulon, C., Martin, S., Padovan, E., Hartmann, U., and Kohler, J. (1996) T cell immune responses to haptens. Structural models for allergic and autoimmune reactions. *Toxicology* 107, 141–151.
- (157) Padovan, E., Bauer, T., Tongio, M. M., Kalbacher, H., and Weltzien, H. U. (1997) Penicilloyl peptides are recognized as T cell antigenic determinants in penicillin allergy. *Eur. J. Immunol.* 27, 1303–1307.
- (158) Weltzien, H. U., and Padovan, E. (1998) Molecular features of penicillin allergy. *J. Invest. Dermatol.* 110, 203–206.
- (159) Naisbitt, D. J., Hough, S. J., Gill, H. J., Pirmohamed, M., Kitteringham, N. R., and Park, B. K. (1999) Cellular disposition of sulphamethoxazole and its metabolites: implications for hypersensitivity. *Br. J. Pharmacol.* 126, 1393–1407.
- (160) Lavergne, S. N., Wang, H., Callan, H. E., Park, B. K., and Naisbitt, D. J. (2009) Danger conditions increase sulfamethoxazole-protein adduct formation in human antigen-presenting cells. *J. Pharmacol. Exp. Ther.* 331, 372–381.
- (161) Oge, M. O., Saide, K., Faulkner, L., Whitaker, P., Peckham, D., Alfirevic, A., Baker, D. M., Sette, A., Pirmohamed, M., Park, B. K., and Naisbitt, D. J. (2015) HLA-DQ allele-restricted activation of nitroso sulfamethoxazole-specific CD4-positive T lymphocytes from patients with cystic fibrosis. *Clin. Exp. Allergy* 45, 1305–1316.
- (162) Daly, A. K., Donaldson, P. T., Bhatnagar, P., Shen, Y., Pe'er, I., Floratos, A., Daly, M. J., Goldstein, D. B., John, S., Nelson, M. R., Graham, J., Park, B. K., Dillon, J. F., Bernal, W., Cordell, H. J., Pirmohamed, M., Aithal, G. P., Day, C. P., and DILIGEN Study, and International SAE Consortium (2009) HLA-B*57:01 genotype is a major determinant of drug-induced liver injury due to flucloxacillin. *Nat. Genet.* 41, 816–819.
- (163) Monshi, M. M., Faulkner, L., Gibson, A., Jenkins, R. E., Farrell, J., Earnshaw, C. J., Alfirevic, A., Cederbrant, K., Daly, A. K., French, N., Pirmohamed, M., Park, B. K., and Naisbitt, D. J. (2013) Human leukocyte antigen (HLA)-B*57:01-restricted activation of drug-specific T cells provides the immunological basis for flucloxacillin-induced liver injury. *Hepatology* 57, 727–739.
- (164) Wuillemin, N., Adam, J., Fontana, S., Krähenbühl, S., Pichler, W. J., and Yerly, D. (2013) HLA haplotype determines hapten or p-i T cell reactivity to flucloxacillin. *J. Immunol.* 190, 4956–4964.
- (165) Wuillemin, N., Terracciano, L., Beltraminelli, H., Schlapbach, C., Fontana, S., Krähenbühl, S., Pichler, W. J., and Yerly, D. (2014) T cells infiltrate the liver and kill hepatocytes in HLA-B(*)57:01-associated floxacillin-induced liver injury. *Am. J. Pathol.* 184, 1677–1682.
- (166) Shubber, Z., Calmy, A., Andrieux-Meyer, I., Vitoria, M., Renaud-Théry, F., Shaffer, N., Hargreaves, S., Mills, E. J., and Ford, N. (2013) Adverse events associated with nevirapine and efavirenz-based first-line antiretroviral therapy: a systematic review and meta-analysis. *AIDS* 27, 1403–1412.
- (167) Sharma, A. M., Li, Y., Novalen, M., Hayes, M. A., and Uetrecht, J. (2012) Bioactivation of nevirapine to a reactive quinone methide: implications for liver injury. *Chem. Res. Toxicol.* 25, 1708–1719.
- (168) Mak, A., and Uetrecht, J. (2015) The Combination of Anti-CTLA-4 and PD1-/- Mice Unmasks the Potential of Isoniazid and Nevirapine To Cause Liver Injury. *Chem. Res. Toxicol.* 28, 2287–2291.
- (169) Lopez, S., Blanca-Lopez, N., Cornejo-Garcia, J. A., Canto, G., Torres, M. J., Mayorga, C., and Blanca, M. (2007) Nonimmediate reactions to betalactams. *Curr. Opin. Allergy Clin. Immunol.* 7, 310–316.
- (170) Alfirevic, A., and Pirmohamed, M. (2010) Drug-induced hypersensitivity reactions and pharmacogenomics: past, present and future. *Pharmacogenomics* 11, 497–499.

- (171) Gibson, A., Ogese, M., Sullivan, A., Wang, E., Saide, K., Whitaker, P., Peckham, D., Faulkner, L., Park, B. K., and Naisbitt, D. J. (2014) Negative regulation by PD-L1 during drug-specific priming of IL-22-secreting T cells and the influence of PD-1 on effector T cell function. *J. Immunol.* 192, 2611–2621.
- (172) Hanzlik, R. P., Fang, J., and Koen, Y. M. (2009) Filling and mining the reactive metabolite target protein database. *Chem.-Biol. Interact.* 179, 38–44.
- (173) Pirmohamed, M., Ostrov, D. a., and Park, B. K. (2015) New genetic findings lead the way to a better understanding of fundamental mechanisms of drug hypersensitivity. *J. Allergy Clin. Immunol.* 136, 236–244.
- (174) Ostrov, D. A., Grant, B. J., Pompeu, Y. A., Sidney, J., Harndahl, M., Southwood, S., Oseroff, C., Lu, S., Jakoncic, J., de Oliveira, C. A. F., Yang, L., Mei, H., Shi, L., Shabanowitz, J., English, A. M., Wriston, A., Lucas, A., Phillips, E., Mallal, S., Grey, H. M., Sette, A., Hunt, D. F., Buus, S., and Peters, B. (2012) Drug hypersensitivity caused by alteration of the MHC-presented self-peptide repertoire. *Proc. Natl. Acad. Sci. U. S. A.* 109, 9959–9964.
- (175) Boerma, J. S., Dragovic, S., Vermeulen, N. P. E., and Commandeur, J. N. M. (2012) Mass spectrometric characterization of protein adducts of multiple P450-dependent reactive intermediates of diclofenac to human glutathione-S-transferase P1–1. *Chem. Res. Toxicol.* 25, 2532–2541.
- (176) Grilo, N. M., Charneira, C., Pereira, S. A., Monteiro, E. C., Marques, M. M., and Antunes, A. M. (2014) Bioactivation to an aldehyde metabolite—possible role in the onset of toxicity induced by the anti-HIV drug abacavir. *Toxicol. Lett.* 224, 416–423.
- (177) Dietz, L., Esser, P. R., Schmucker, S. S., Goette, I., Richter, A., Schnölzer, M., Martin, S. F., and Thierse, H. (2010) Tracking human contact allergens: from mass spectrometric identification of peptide-bound reactive small chemicals to chemical-specific naive human T-cell priming. *Toxicol. Sci.* 117, 336–347.
- (178) Aleksic, M., Pease, C. K., Basketter, D. A., Panico, M., Morris, H. R., and Dell, A. (2008) Mass spectrometric identification of covalent adducts of the skin allergen 2,4-dinitro-1-chlorobenzene and model skin proteins. *Toxicol. In Vitro* 22, 1169–1176.
- (179) Golizeh, M., and Sleno, L. (2013) Optimized proteomic analysis of rat liver microsomes using dual enzyme digestion with 2D-LC-MS/MS. *J. Proteomics* 82, 166–178.
- (180) Meng, X., Maggs, J. L., Usui, T., Whitaker, P., French, N. S., Naisbitt, D. J., and Park, B. K. (2015) Auto-oxidation of Isoniazid Leads to Isonicotinic-Lysine Adducts on Human Serum Albumin. *Chem. Res. Toxicol.* 28, 51–58.
- (181) Aloysius, H., Tong, V. W., Yabut, J., Bradley, S. A., Shang, J., Zou, Y., and Tschirret-Guth, R. A. (2012) Metabolic activation and major protein target of a 1-benzyl-3-carboxyazetidine sphingosine-1-phosphate-1 receptor agonist. *Chem. Res. Toxicol.* 25, 1412–1422.
- (182) Ballard, T. E., Dahal, U. P., Bessire, A. J., Schneider, R. P., Geoghegan, K. F., and Vaz, A. D. N. (2015) A tag-free collisionally induced fragmentation approach to detect drug-adducted proteins by mass spectrometry. *Rapid Commun. Mass Spectrom.* 29, 2175–2183.
- (183) Deng, Z., Zhong, D., and Chen, X. (2012) Identification of modification sites on human serum albumin and human hemoglobin adducts with houttuynin using liquid chromatography coupled with mass spectrometry. *Biomed. Chromatogr.* 26, 1377–1385.
- (184) Lin, H., Kenaan, C., and Hollenberg, P. F. (2012) Identification of the residue in human CYP3A4 that is covalently modified by bergamottin and the reactive intermediate that contributes to the grapefruit juice effect. *Drug Metab. Dispos.* 40, 998–1006.